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ÄKTAcrossflow

Method Handbook



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1 Introduction

1.1 Cross flow filtration and membrane filters

1.1.1 What is Cross Flow Filtration?

Cross Flow Filtration (CFF) is a filtration process in which the feed solution tangentially passes along the surface of the filter. A pressure difference across the filter is used to drive those components through the filter that are smaller than the pores. Components larger than the filter's pores are retained and pass across the membrane surface, flowing back to the feed reservoir (Fig. 1-1). The key feature of CFF is the cross flow. The cross flow of fluid along the membrane surface sweeps away the build up of material deposits on the filter surface and prevents the filter from fouling quickly. CFF is simple in concept, but its proper execution requires detailed knowledge and good filtration technique.

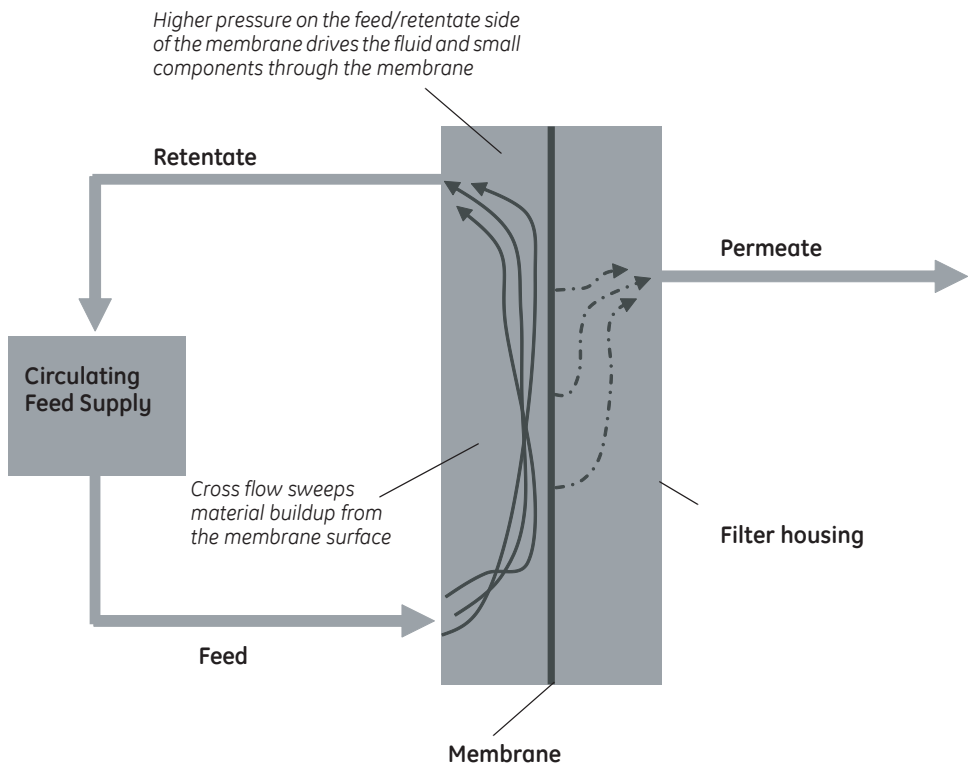


Fig 1-1. The fundamental concept and terminology of cross flow filtration.

1.1.2 How does CFF differ from conventional filtration?

CFF differs from conventional filtration in two ways. First, CFF filters use membranes exclusively, while conventional filtration may use membranes, paper, or nonwovens to separate components in a feed stream. Secondly, the feed in a CFF filter circulates across the membrane surface multiple times. In conventional filtration, the feed is directed at the surface of the filtration media and does not circulate. Hence, as the filter cake builds up, the filtration characteristics change, the fluid flow decreases markedly, and eventually the filtering ends. Typically, the filtration membrane is single use at the laboratory scale.

1.1.3 Microfiltration and ultrafiltration

Although cross flow filtration encompasses a wide range of membrane technologies, for the purpose of this handbook, CFF can be divided into two classes: microfiltration and ultrafiltration. Microfiltration filters have larger pores than their ultrafiltration counterparts.

1.1.4 Microfiltration filters

Membranes with 0.1 μm to 10 μm pore size ratings are classified as microfilters, however in CFF the practical pore size ranges from 0.1 μm to 1 μm . Membranes with 0.65 μm , 0.45 μm , 0.2 μm , and 0.1 μm pore size ratings are used for separation of cultured cells from the growth medium (broth), as well as for sterilization, and contaminant and particle removal in numerous biopharmaceutical processes.

1.1.5 Ultrafiltration filters

Membranes with 1,000 to 1,000,000 Daltons nominal molecular weight cutoff (NMWC) are called ultrafilters. These membrane are used for concentrating and fractionating protein streams, virus concentration, desalting and buffer exchange. The objective of most ultrafiltration processes is to retain and fractionate soluble macromolecules such as proteins, while allowing liquid and unwanted smaller molecules to pass such as salts, amino acids, and mono- or di-saccharides (Fig. 1-2).

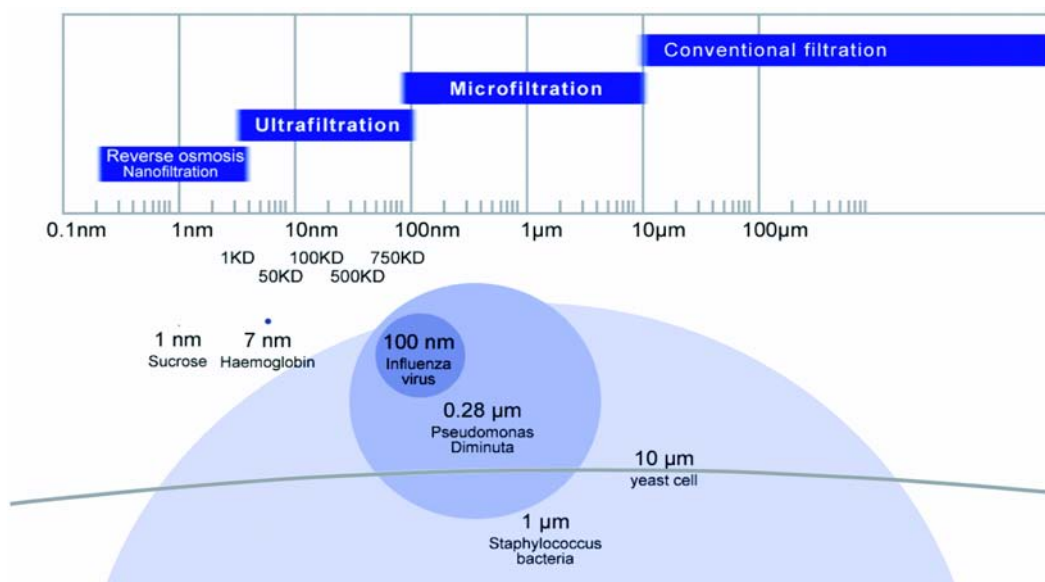


Fig 1-2. Relative size of CFF feed components and operational scales for filtration.

1.2 CFF terminology

Feed	The starting solution or suspension that is pumped to the filter for separation.
Permeate	Any components of the feed that pass through the membrane.
Retentate	Any component that does not pass through the membrane, but instead circulates through the retentate line back to the feed tank.
Cross flow rate	The rate of flow across the membrane surface. Higher cross flow rates help sweep away the debris that forms on the surface of the filter. Cross flow rate is most often measured at the retentate outlet.
Flux	Flux represents the volume of solution flowing through a given membrane area during a given time and commonly expressed as LMH (liters per square meter of membrane per hour). Flux is a key process criterion directly affecting production rate and determining filter performance.
ΔP	The pressure differential between the feed and retentate lines. The differential pressure equals the feed pressure minus the retentate pressure.
Transmembrane pressure (TMP)	The pressure that drives components of the feed solution through the membrane. As a key process variable, TMP can help drive the process or if not controlled properly, blind the filter, resulting in low uncontrolled flux rates. TMP is calculated as: $[(\text{feed pressure} + \text{retentate pressure}) \div 2] - \text{permeate pressure}$
Cell processing	The broad term used to describe the processes of cell harvesting, cell clarification, and lysate clarification. (Also called upstream processing.)
Cell harvesting	A cell processing application that separates cells from fermentation broth with the goal of recovering the cells.
Cell clarification	A cell processing application that separates cells from the fermentation broth with the goal of recovering the broth and a protein(s) in the broth.
Lysate clarification	A cell processing application that separates proteins from the cell lysate.

Concentration/ diafiltration	The process of concentrating and buffering proteins in preparation for chromatographic processing or for final formulation of an end product.
Hollow fiber cartridge	Hollow fiber cartridges consists of bundles of cylindrical fibers with lumen diameters ranging from 0.25 to 3 mm. Feed flows through the lumen under pressure and the permeate passes from the inside to the outside of the hollow fibers.
Membrane cassette	Membrane cassettes consist of layers of flat sheets of membrane sandwiched together, often with a spacer between the layers.
Shear rate	The ratio of velocity and flow section expressed in units of sec^{-1} . The shear rate for a hollow fiber cartridge is based on the flow rate through the fiber lumen. While excessive shear (excessive feed stream flow rate) can potentially damage cells and proteins, higher shear rates generally result in flux improvements.
Concentration factor	The concentration factor is the ratio of the initial feed volume to retentate volume after separation. For example, if the initial feed volume is 100 l and the final retentate volume is 20 l, the concentration factor is 5x.
Diafiltration	Diafiltration is a unit operation that incorporates ultrafiltration membranes to remove salts or other microsolute from a solution. Small molecules are separated from a solution while larger molecules remain in the retentate. In general, microsolute are easily washed through the membrane so that for a fully permeated species approximately three volumes of diafiltration solution will eliminate 95%-99% of the microsolute.

Table 1-1. CFF key terminology. The appendix includes a glossary that defines additional terms and concepts.

1.3 Applications overview

CFF is used in research, product development, and production in the biopharmaceutical, industrial, and medical industries (Table 1-2).

Industry	Application	Comments
Biopharmaceuticals cell processing (upstream processing)	Harvesting cells from fermentation broths	Cells harvested include bacteria, insect Cell is the product of interest Most cells are insensitive to shear Wide range of membranes will work Cells are large compared to pores. A broad range of microfiltration membrane may be used.
	Separating proteins from fermentation broths	Separating proteins from intact cells and potential cell debris Protein in broth is product of interest Usually mammalian cells Shear damages cells and proteins Yeast cells are difficult to process Membrane selection is the key to success Open membranes can lead to turbidity and process control issues
	Separating proteins from cell lysates	Protein in lysate is product of interest Excessive shear damages protein Membrane selection is key to success Open membranes can lead to turbidity and process control issues
Biopharmaceuticals Concentration/ Diafiltration (downstream processing)	Concentrating and buffering target molecules	Products can be shear sensitive Membrane selection is key
	Final formulation of bulk drug substance	Includes concentrating protein and exchanging buffer to final product formulation

Table 1-2. Typical uses of cross flow filtration.

1.3.1 Cell processing and concentration/diafiltration

The terms cell processing and concentration/diafiltration are used in this handbook to differentiate between the main types of biopharmaceutical applications (Table 1-3).

Main types of CFF applications in the biopharmaceutical industry		
1. Cell processing		
Cell harvesting	Cell clarification	Lysate clarification
Recovers cells from fermentation broth	Recovers cells, all fragments, and other particles from the target protein in the cell-broth mixture	Remove the cell fragments and macrosolutes from the target protein
Cells in the retentate are the product of interest	Protein in the permeate is the product of interest	Protein in the permeate is the product of interest
2. Concentration / Diafiltration		
Concentrate and buffer protein		
Protein in the retentate is the product of interest		

Table 1-3. Application terminology.

1.4 *Technical parameters*

An optimized CFF process starts with a characterization of the feed material as follows:

- Temperature sensitivity of the feed material
- pH stability range of the target molecule
- Sensitivity of the target molecule or cell to shear forces
- Target molecule solubility
- Availability of a suitable assay for monitoring yield and finished product activity
- Is it possible to concentrate the feed to the target concentration given the starting volume and the system's working volume?
- Will increases in viscosity due to cell mass concentration exceed the capability of the CFF system?

1.5 Approximate times for completing CFF runs

Typically, the time for completing a cross flow filtration run using ÄKTAcrossflow™ system can range from 3 to 8 hours. A run can be divided into three parts: preparing the filter and system for processing, conducting the separation/concentration/diafiltration process, and cleaning and flushing the system and filter for storage (Table 1-4). ÄKTAcrossflow completes many of these tasks automatically. In addition, it fully monitors and records process parameters throughout the run, freeing operators to perform other tasks.

Function	Steps	Time required
Preparing the filter and system for processing	Sanitization (optional) Rinsing Water flush Water flux test Buffer conditioning	Up to 120 minutes
Product processing	Cell processing and washing or Protein concentration and diafiltration	Time dependent on surface area applied per feed volume, target concentration factor, and diafiltration exchange volume
Cleaning and flushing the system	Buffer flush Cleaning/sanitization Water flush Water flux test Storage	Up to 120 minutes

Table 1-4. Typical times for completing CFF procedures using ÄKTAcrossflow system.

1.6 Water quality requirements

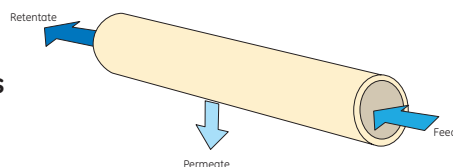
To prevent plugging the pores of the membrane filter, always use deionized water, ultrafiltered water (10,000 NMWC), or water-for-injection when rinsing or flushing, when making up cleaning solutions or when adding water for diafiltration of process fluids.

1.7 Membrane filtration devices

The membrane inside a membrane filter performs the separation by size sieving the components in the feed stream. Hence membrane characteristics represent a key variable in selecting a CFF filter. Both flat sheet cassettes and hollow fiber cartridges are available from GE Healthcare.

Filter Type Selection

Open Channel Hollow Fiber Cartridges



Screen Channel Flat Sheet Cassettes

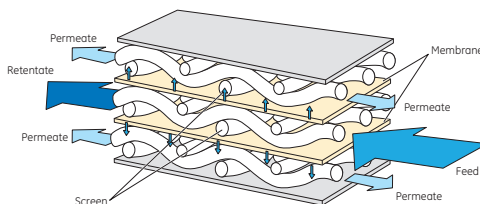


Fig 1-3. Mechanism of action for hollow fiber cartridges and flat sheet cassettes.

Filter characteristics that influence performance include the following:

- Fiber length and lumen diameter (hollow fiber cartridges)
- Flow path length and channel height (cassettes)
- Membrane surface area
- Pore size
- Material in membrane

1.7.1 Fiber length and lumen diameter (cartridges)

Fiber length and lumen diameter in Start AXM and Start AXH cartridges are controllable variables that influence a CFF process. Available fiber lengths are 30 cm and 60 cm. Fiber lumen diameter for these cartridges range from 0.5 mm to 1 mm.

1.7.2 Flow path length and channel height (cassettes)

Kvick Start™ filters available for ÄKTAcrossflow system use two layers of flat sheet membrane separated by a screen. The flow path length is 17 cm. The channel height equals the thickness of the screen.

1.7.3 Membrane surface area

ÄKTAcrossflow system works best with ultrafiltration cassettes having 50 cm² to 150 cm² of membrane surface area, and hollow fiber microfiltration cartridges having 50 cm² of membrane surface area. When choosing the membrane surface area, consideration must be given to the starting volume of the product, the nature of the product, the desired processing time, and operating pressures. Typical values for the selection of the membrane surface area are as follows:

- 30 to 100 liters of feed per square meter of membrane surface area for microfiltration
- 100 to 200 liters of feed per square meter of membrane surface area for ultrafiltration

1.7.4 Pore size

Membrane pore size determines the size of the particles or molecules that pass through the membrane. The pores in a membrane vary in size, so the size distribution of the pores determines the sharpness of the separation.

Ultrafiltration

The pore size of ultrafiltration membranes is expressed as nominal molecular weight cutoff (NMWC). ÄKTAcrossflow ultrafiltration membrane filters are available in both hollow fiber cartridge and membrane cassette formats.

Microfiltration

The pore size for microfiltration cartridges is expressed in microns. ÄKTAcrossflow cartridges have average pore size ratings from 0.1 µm to 0.65 µm.

Guidelines for selecting membrane pore size are found in later sections of this handbook. The automation and minimum operating volume of ÄKTAcrossflow system make it easy to screen different pore sizes to find the best performing membrane for a given application. In Appendix A you will find information of all micro filtration cartridges and ultra filtration membrane filters supplied by GE Healthcare.

1.8 Membrane structure

1.8.1 Ultrafiltration membrane

Ultrafiltration membrane from GE Healthcare has a macrovoid-free structure which provides high tensile strength, high temperature resistance and stable performance throughout the service life (Fig. 1-4). The membrane structure includes a skin layer and a supporting substructure.

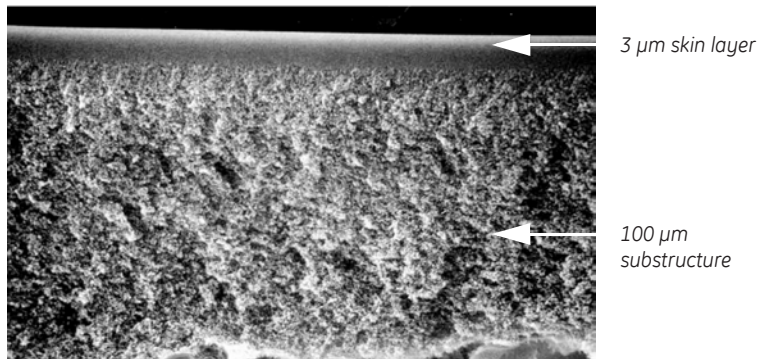


Fig 1-4. Scanning electron micrograph showing the structure of a hollow fibre ultrafiltration membrane from GE Healthcare.

1.8.2 Microfiltration membrane

Microfiltration hollow fiber membranes from GE Healthcare have a uniform, microporous, sponge-like structure.

1.9 Membrane filter design

1.9.1 Kwick Start cassettes

Kwick Start cassettes include a feed port, a retentate port, and two permeate ports. The ports use 5/16"-24 UNF female fittings for connections (Fig. 1-5). Adaptors enable the connection of the ports to male Luer-Lok fittings on laboratory tubing if desired.

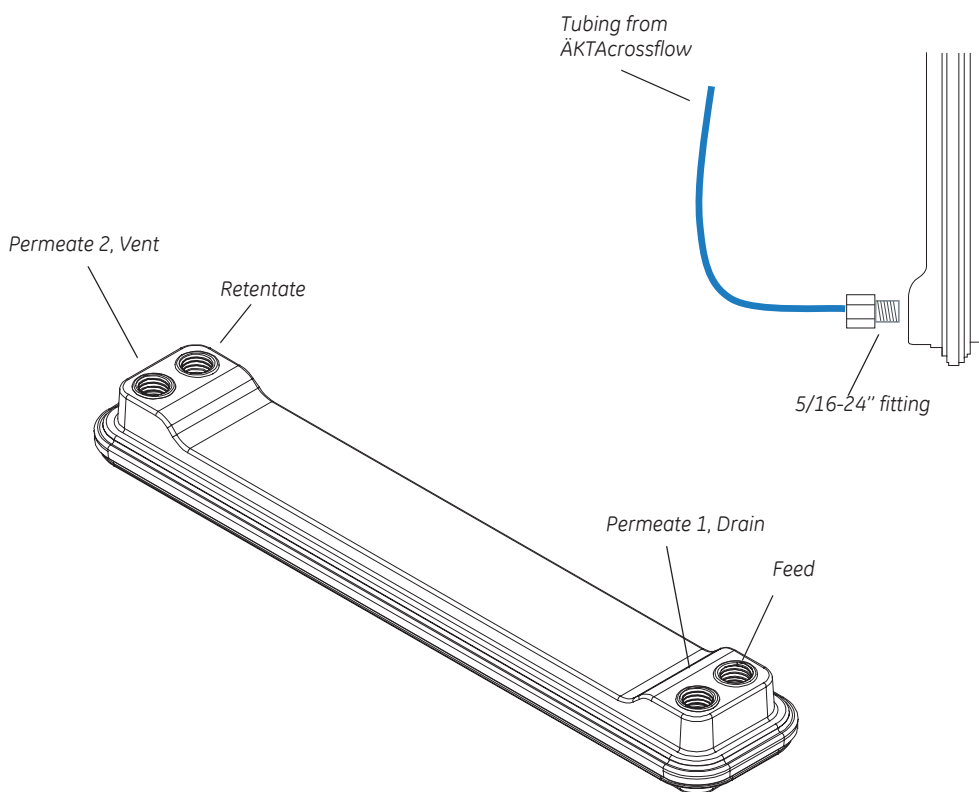


Fig 1-5. Kwick Start cassette.

1.9.2 Hollow fiber cartridges

ÅKTAcrossflow hollow fiber cartridges include a feed port, a retentate port, and two permeate ports. The ports on the Start AXM and Start AXH cartridges use 5/16"-24" UNF female fittings for quick and easy connection to ÅKTAcrossflow system tubing (Fig. 1-6).

Microfiltration cartridges are shipped dry and can be used immediately after wetting out with buffer. Ultrafiltration cartridges contain an alcohol/glycerin storage solution, and must be thoroughly rinsed before use.

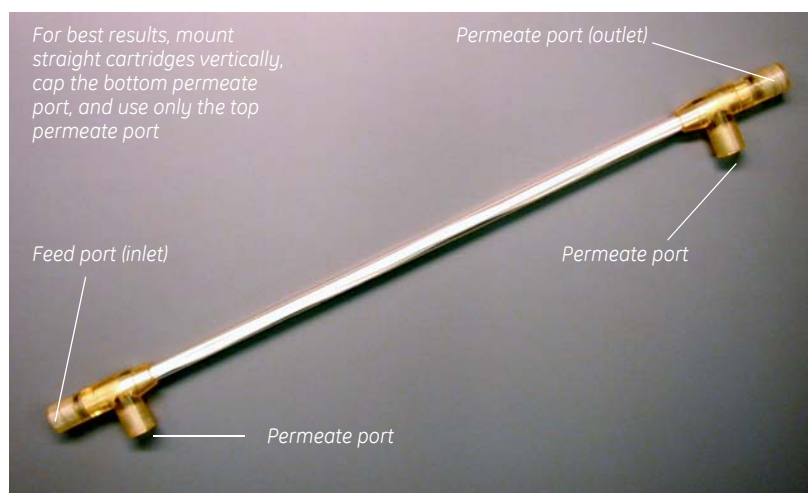
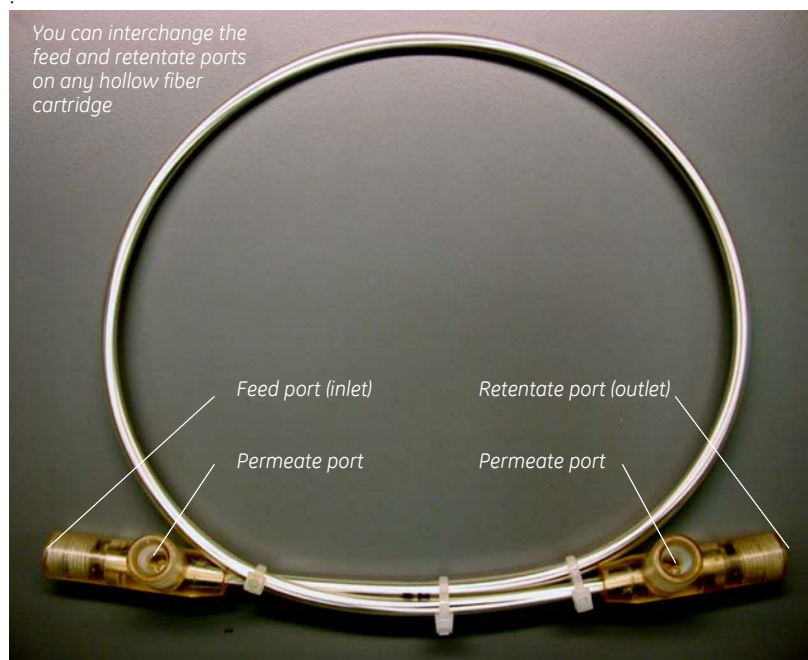


Fig 1-6. Start AXM and Start AXH cartridges.

1.10 CFF filter life cycle

The long-term stability of a used filter may vary depending on many factors including the following:

- Components in the process solution
- Processing conditions
- Aggressiveness of the cleaning protocols
- Handling and storage conditions

Typically the performance of the filter is checked before and after use by measuring the rate of water flow through the membrane under controlled conditions. When the performance of the filter drops to unacceptable levels, the filter should be replaced (Fig. 1-7). At the laboratory scale, some users dispose of membrane filters after each use. This avoids the use of cleaning chemicals and their disposal, cleaning time, and the possibility for cross contamination from the filter.

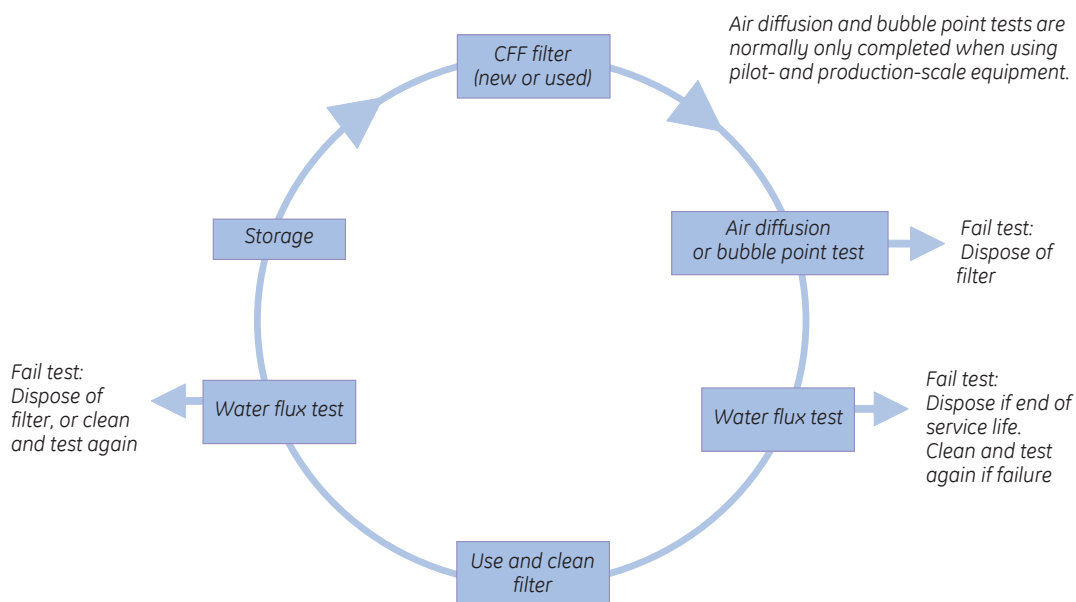


Fig 1-7. Life cycle of membrane filters.

1.11 Membrane filter specifications

The following sections describe technical aspects of membrane filters. The appendix provides detailed specifications.

1.11.1 Materials of construction

Three desirable characteristics of membrane filters are mechanical strength, chemical and physical compatibility, and low extractables and toxicity ratings. The membrane materials (polyethersulfone and polysulfone) used in ÄKTAcrossflow membrane filters offer broad pH and thermal stability, and provide good chemical compatibility with many bioprocess fluids and cleaning solutions.

1.11.2 Kwick Start cassettes

The construction materials for Kwick Start cassettes are as follows:

- Fluid path
 - Inner plates - Polyester copolymer
 - Membrane screen - Polypropylene
 - Membrane - Polyethersulfone
 - Port sealer - Solvent-free urethane (meth) acrylate blend
 - Luer-Lock™ adapters - Polypropylene
 - Luer-Lock adapter gasket - EPDM (Ethylene propylene diene monomer)
- Housing - Epoxy
- Wetting fluid - 0.1 - 0.2N sodium hydroxide and 20 - 22% (w/v) glycerine

1.11.3 Start AXM and Start AXH cartridges

The construction materials for Start AXM and Start AXH cartridges are as follows:

- Housing - Polysulfone
- Membrane - Polysulfone
- Luer-Lok fittings - Polycarbonate
- Potting - Epoxy
- Wetting fluid - Glycerine in case of UF membranes.
 - MF membranes are delivered dry

1.12 Testing procedures

1.12.1 Water flux test

The water flux test measures the flow rate of water through the membrane under controlled conditions. The flow rate provides an indication of the performance capability of the membrane. By tracking the water flux measurements over time, it is possible to determine the effectiveness of cleaning cycles, and determine when a cassette reaches the end of its service life. A filter will normally lose up to 20 percent of its performance, as measured by the water flux test, after its first use and cleaning. The performance level should remain stable from that time forward. Water flux testing is usually carried out when the filter is new and after each use or cleaning cycle. ÄKTAcrossflow system software contains a method to automatically measure the water flux of the filter and calculate and plot the results in LMH/bar. Details of the water flux test procedure can be found in ÄKTAcrossflow User Reference Manual.

1.13 Quality assurance and documentation

Quality assurance documentation and evidence of consistent performance (process validation) are key process requirements when using CFF systems and filters in biopharmaceutical applications.

1.13.1 Hollow fiber cartridges

GE Healthcare supplies each hollow fiber cartridge with a Certificate of Test stating the model number, batch number, and test results from quality assurance testing. Each cartridge is individually tested at the factory for fiber and cartridge integrity.

All cartridges and cartridge components meet the specifications of the following tests:

- USP Class VI Pastics 70°C
- Hemolysis-Rabbit Blood (Direct Contact)-ISO 10993
- L929 MEM Elution Test-ISO 10993

1.13.2 Cassettes

GE Healthcare supplies each cassette with a Certificate of compliance. The certificate of compliance includes the lot and serial number and states the cassette has been manufactured and tested in accordance with standard operating procedures and is certified to meet the specifications established by GE Healthcare.

All cassettes and cassette components meet the specifications of the following tests:

- USP Class VI Plastics 70°C
- Hemolysis-Rabbit Blood (Direct Contact)-ISO 10993
- L929 MEM Elution Test-ISO 10993

2 ÄKTAcrossflow system components and software

2.1 System overview

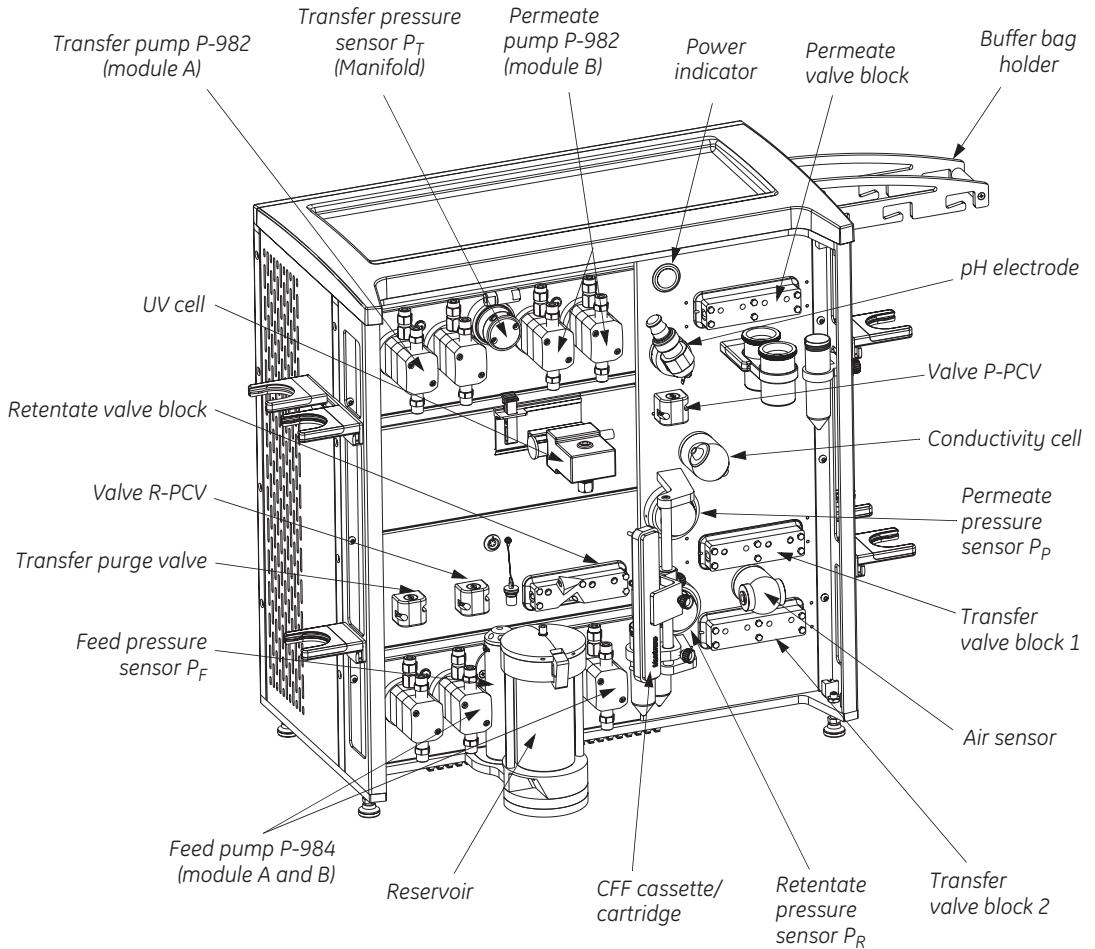


Fig 2-1. ÄKTAcrossflow instrument showing key components.

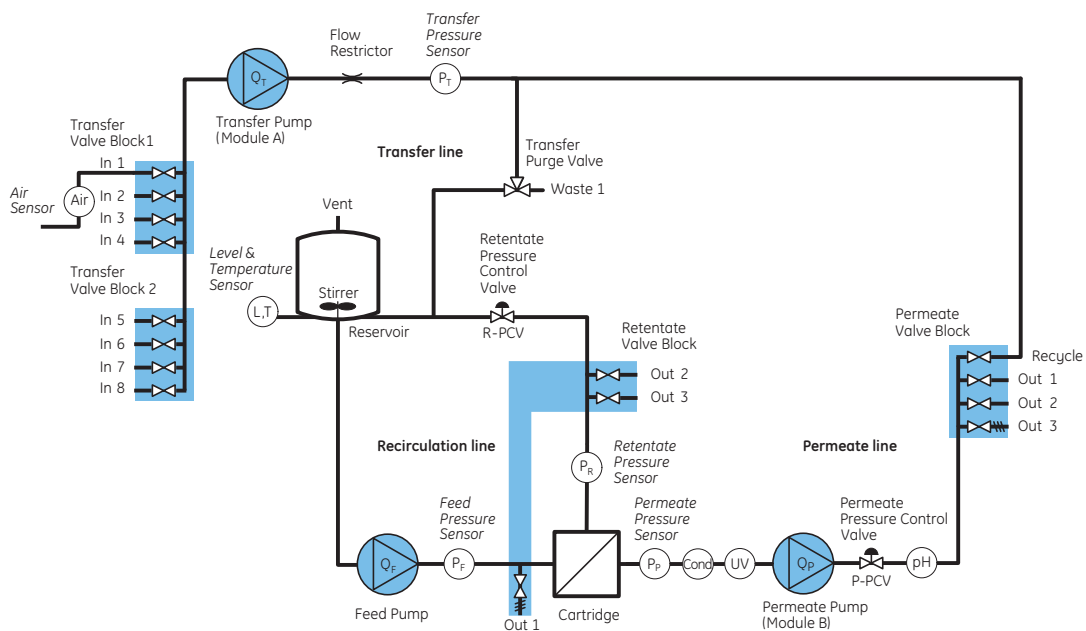


Fig 2-2. Diagram of the general flow scheme in ÄKTAcrossflow.

2.2 ÄKTAcrossflow system components

2.2.1 Pumps

The pumps of ÄKTAcrossflow system are high-precision, metering pumps. The pump heads have a sanitary design with a self-contained rinsing system to prevent contamination and pump damage. For more information regarding shear effects on cells and proteins, see Appendix C. The low shear design ensures that sensitive cellular material is not damaged during operation. Furthermore this design guarantees negligible heat transfer from the pump heads to the process fluid.

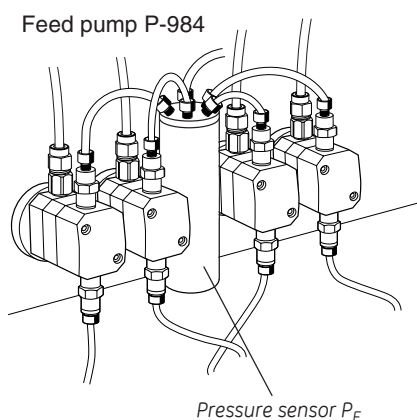


Fig 2-3. Feed pump P-984.

Pump P-982 and Pump P-984

Pump P-982 and P-984 are high performance laboratory pumps for use in applications where accurately controlled liquid flow is required. Twin reciprocating pump heads work in unison to deliver a smooth and pulsation free flow.

P-982 is used as the transfer pump (module A) and as the permeate pump (module B). P-984 is used as the feed pump (module A and B)

Pump P-982 features:

- Four pump heads arranged in two pairs of two
- Pressure range 0-520 kPa (5.2 bar, 75.4 psi)
- Flow rate range 0.1-200 ml/min

Pump P-984 features:

- 4 pump heads
- Pressure range 0-520 kPa (5.2 bar, 75.4 psi)
- Flow rate range 1-600 ml/min

2.2.2 Pump heads

Each pump head has an inlet check valve and an outlet check valve for the liquid flow. In addition, each pump head has an outlet check valve for the rinsing flow system. The individual pump heads are actuated in opposite phase to each other by microprocessor-controlled individual stepper motors. The synchronization of the pump heads generates a constant flow with low pulsation. For the feed pump this synchronization is optimized to yield a low pulsation flow at the inlet and outlet. However the pump heads of the permeate pump are synchronized such that the flow at the pump inlet has low pulsation, and for the transfer pump the pump outlet has low pulsation. Pressure and flow at the permeate side of the filter cartridge can thus be controlled with a high degree of accuracy. The pump heads are made from titanium alloy.

2.2.3 Piston rinsing system

Leakage between the pump chamber and the drive mechanism is prevented by a seal. The seal is continuously lubricated by the presence of buffer. In order to prevent any deposition of salts from aqueous buffers and other organic compounds on the pistons, and to prolong the life of the seals, the pump has a piston rinsing system. The low pressure chamber situated behind the piston can be flushed continuously with 10 mM sodium hydroxide in 20% ethanol. A check valve in the system ensures that there is a continuous flow of rinsing fluid.

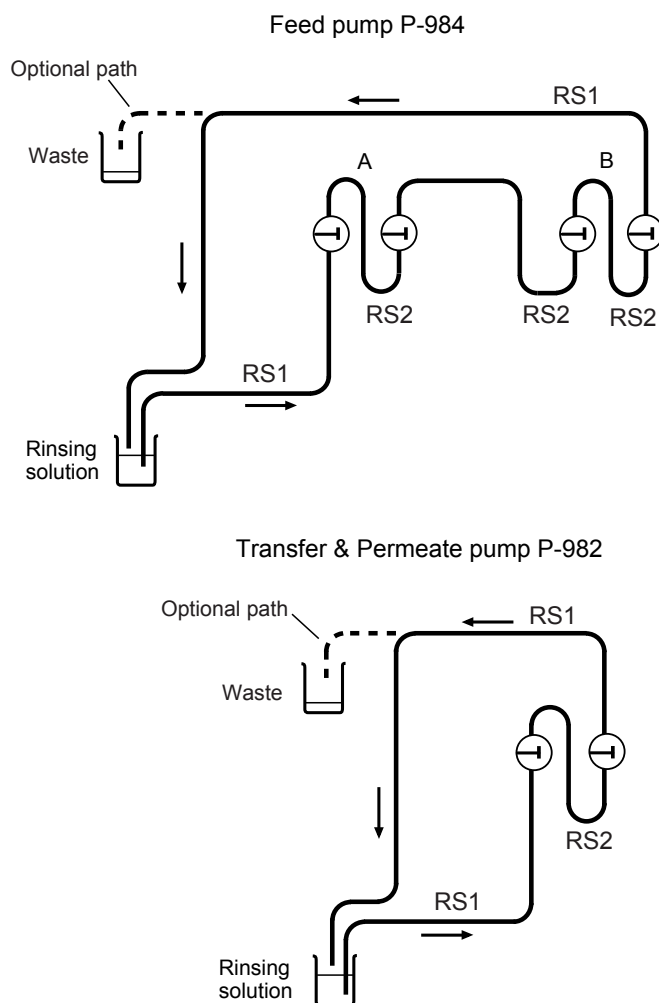


Fig 2-4. Piston rinsing system: Feed pump P-984 and Transfer and Permeate pump P-982.

2.2.4 Reservoir

The reservoir contains the liquid/sample to be processed. It provides a gentle but efficient mixing of the process liquid with returning retentate and additional liquid added via the transfer line. Permeate may be recycled into the reservoir to achieve steady-state conditions during process development studies.

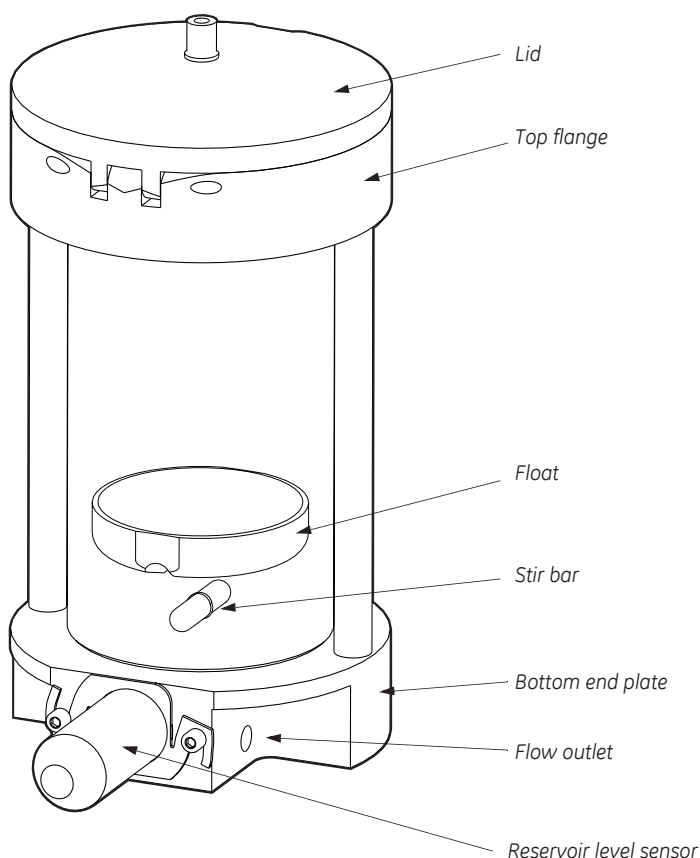


Fig 2-5. Reservoir 350 ml.

A magnetic stir bar in the bottom of the reservoir ensures uniform mixing between the bulk fluid, the retentate returned from the filter and liquid added via the transfer line.

An integrated level and temperature sensor continuously monitors and reports the retentate liquid volume and the temperature of the liquid fed into the filter device. The level sensor can also be used to protect the filter against the introduction of air.

The reservoir is equipped with a float to prevent vortex formation and foaming so that operation at lowest circulation volume with high low rate is facilitated.

There are two sizes of reservoir:

- 350 ml (375 ml without float)
- 1100 ml (1200 ml without float)

2.2.5 Liquid connections

Each reservoir has connections for the liquid flow positioned at the reservoir bottom end plate. There is one outlet for delivering liquid to the feed pump via a manifold, and the outlet is placed off-centre at the bottom of the reservoir to prevent vortex formation. The retentate return is positioned such that the liquid is injected tangentially to the bottom surface.

2.2.6 Magnetic stir bar

The reservoir is mounted on a reservoir holder which contains a motor unit for a magnetic stir bar. The stir bar can be used with both reservoirs to improve mixing characteristics.

Recommended stir bar dimensions are:

- 375 ml reservoir: length of stir bar 30 mm, diameter 6 mm (max. diameter at pivot ring: 7 mm)
- 1200 ml reservoir: length of stir bar 35 mm, diameter 6 mm (max. diameter at pivot ring: 8mm)

The appropriate mixing rate is a function of application and retentate volume and can be adjusted by the control software. As default, the UNICORN™ control software adjusts the mixing rate automatically depending on the actual retentate volume. At low retentate volume, the stir bar and the float will be in contact such that the stir bar will rotate the float. Under these conditions a low mixing rate is selected as default by the control software. At higher retentate volume where the float is not in contact with the stir bar, the user can select a higher mixing rate.

The following mixing rates are recommended as maximum mixing rates that will ensure sufficient mixing for all conditions:

- 375 ml reservoir: 200 rpm
- 1200 ml reservoir: 300 rpm

2.2.7 Materials

The reservoir consists of the following material:

- Glass tube - Borosilicate
- Bottom end plate, top flange and lid - Polyetherimide
- Sealing lid - Thermoplastic elastomer
- Float - Polypropylene
- Stir bar- Polytetrafluoroethylene

2.3 Sanitary design

ÄKTAcrossflow has been designed to allow effective sanitization using 1M sodium hydroxide (NaOH) as a sanitizing agent. Sanitization is the use of a chemical agent to reduce a microbial population to an acceptable, predetermined level. Microbial challenge tests are used to evaluate the efficiency of the sanitizing agent.

A study including two challenging organisms has been carried out. The system was subjected to a high level of microbial challenge (1×10^6 Colony Forming Units CFU/ml). The results show that the method used efficiently reduced the numbers of viable organisms and was sufficient for sanitization.

2.3.1 Valves

The liquid flow in ÄKTAcrossflow system is controlled by valves of different functionality:

- Four membrane valve blocks of stepper motor actuated valves with open/close functionality.
- Two pressure control valves (R-PCV and P-PCV); lever actuated.
- One 2-way switch valve (transfer purge valve); lever actuated.

All valves are sanitary designed with EPDM membranes for high chemical resistance.

Membrane valve block

The valves are located in valve blocks to minimize hold up volumes. A valve block consists of a connection block containing the ports and membranes, and a mechanical housing containing the stepper motor, cams and actuating pistons. The valve blocks have different numbers of inlet and outlet ports depending on their location in the flow path (see flow diagram).

There are four different types of membrane valve block:

- Inlet valves T-VB-In: 1-4
- Inlet valves T-VB-In: 5-8
- Outlet valves R-VB-Out: 1 (safety valve), 2, 3
- Outlet valves P-VB-Out: recycle, 1, 2, 3 (safety valve)

Two of the outlet valves, R-VB-Out 1 and P-VB-Out 3, have built in safety valve functionality with an opening pressure 7 bar (102 psi).

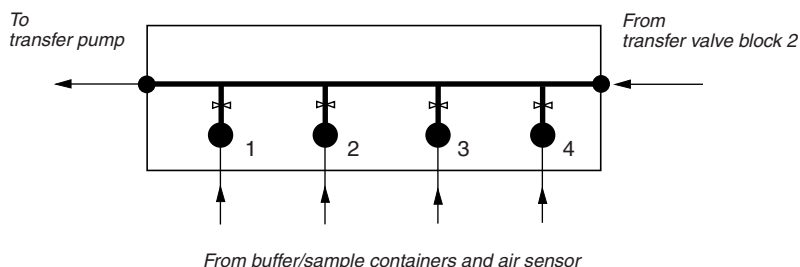


Fig 2-6. Valve block.

Retentate control valve R-PCV

The retentate control valve R-PCV is used to accurately control the retentate pressure over the pressure range 0.1-5.2 bar. In this way the transmembrane pressure (TMP) can be adjusted. In addition, the R-PCV can operate as an open/close valve in product recovery and system cleaning procedures.

Permeate control valve P-PCV

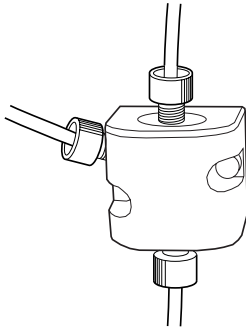
The main function of the permeate control valve P-PCV is to control the pressure downstream of the permeate pump in order to ensure accuracy in the permeate flow rate.

To ensure proper operation of the check valves, the pressure downstream of the pump must be greater than the pressure upstream of the pump. The P-PCV valve is controlled by the software such that it will always maintain a higher pressure downstream of the pump.

2-way transfer purge valve

The transfer purge valve directs the liquid flow either from the transfer line or the permeate recycle line to the reservoir (default) or to waste.

2-way transfer purge valve



Pressure control valves R-PCV and P-PCV

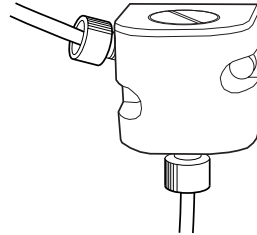


Fig 2-7. Two-way transfer purge valve and pressure control valves R-PCV and P-PCV.

The lever actuated valve units have an EPDM encapsulated lever which is actuated by a solenoid to open or close a flow path. The solenoid adjusts the force of the lever against the flow through the inlet port. This novel and robust design results in the pressure upstream of the valve being maintained irrespective of changes in flow rate, in contrast to conventional control valves.

2.3.2 Flow restrictor in the transfer line

A flow restrictor is positioned downstream of the transfer pump in order to ensure a proper operation of the check valves at the pump heads, and thus accuracy in the transfer flow rate. The restrictor generates a constant back pressure of 3 bar.

2.3.3 Detectors and monitors

ÄKTAcrossflow is equipped with detectors for continuous in-line measurement of pressure, temperature, pH, conductivity and UV absorbance, for accurate and reliable monitoring. The flow cells for UV, conductivity and pH in the permeate line are situated close together to minimize volume and time delay between components. The flow cells are easily accessible from the front panel to facilitate maintenance.

pH measurement

The pH electrode is positioned downstream of the pressure control valve P-PCV. The pH electrode is optimized for continuous pH measurement in ÄKTAcrossflow path. The electrode is of the sealed combination double junction type with a glass tip and the cell holder is made of titanium. The pH monitor provides pH measurement in the range 1-14 (2-12 within specification) and can be used for example to monitor buffer exchange during diafiltration.

UV measurement

The UV cell is normally positioned after the conductivity cell in the permeate line, but it can be moved to the retentate side if required. It is designed for continuous measurement of UV absorbance and provides high performance detection for the wavelengths 214, 254 and 280 nm. The UV cell housing is made of PEEK and other wetted parts are made of glass and titanium. The UV cell is used for measuring the UV absorbance of the permeate. This information is used to ensure protein rejection during ultrafiltration/diafiltration, and also to monitor applications in cell processing.

Conductivity measurement

The conductivity cell is positioned after the permeate pressure sensor in the permeate line. The conductivity cell is useful for measuring, for example, buffer exchange during diafiltration. The cell also contains a temperature sensor. Temperature variations influence the conductivity, and in some applications where precise conductivity values are required it is possible to program a temperature compensation factor that recalculates the conductivity relative to a set reference temperature. The measurement range of the conductivity cell is 1 $\mu\text{S}/\text{cm}$ to 250 mS/cm .

2.3.4 Pressure sensors

ÄKTAcrossflow system is equipped with four pressure sensors.

Pressure sensor	Location
Pf	Close to the CFF filter in the feed line to measure the feed pressure.
Pr	Close to the CFF filter in the retentate line to measure the retentate pressure.
Pp	Close to the CFF filter in the permeate line to measure the permeate pressure.
Pt	Upstream the reservoir, and is mainly used to measure the pressure in the reservoir for safety reasons.

Table 2-1. Pressure sensor location.

To protect the system, pressure limits can be set in UNICORN for the sensors Pf, Pr and Pp. The pressure sensors have a pressure range of 0-10 bar (1 MPa, 145 psi). The pressure sensor housing is made of PEEK. Other wetted parts are made of titanium and stainless steel.

2.3.5 Reservoir level sensor

The reservoir level sensor is located in the reservoir bottom end plate. It is a highly sensitive pressure sensor that continuously reports the hydrostatic pressure in the reservoir, and thus the weight of the retentate, to the control software. These data are then transformed to information on the retentate liquid volume. The level sensor has also the function of a low volume alarm for the reservoir. The level sensor is used to calibrate the volume of ÄKTAcrossflow system during start up, and in addition it ensures efficient product removal at the end of the filtration process by protecting the filter against the introduction of air. The level sensor has a pressure range of 0-100 mbar (10 kPa, 1.45 psi).

A temperature sensor is integrated with the reservoir level sensor and allows for continuous temperature measurement of the liquid feed to the CFF cassette/cartridge.

2.3.6 Air sensor

The air sensor is located in the flow path for the sample inlet. It is designed to continuously monitor for air bubbles and ensure that the maximum volume of external feed can be transferred into the system without any risk of introducing air into the transfer line. When air is detected the system is paused or an action is performed that has been set in the method. Avoiding air in the transfer line is important to ensure the high volume accuracy of the transfer pump and thereby the accuracy of the retentate volume content.

2.4 UNICORN

ÄKTAcrossflow system is controlled and monitored by UNICORN™ software. UNICORN is a complete package for control and supervision of biotechnical systems. It consists of control software, and where applicable a controller card or interface unit for interfacing the controlling PC to the liquid handling module.

2.4.1 Liquid chromatography system version

UNICORN can be used with a number of systems, including ÄKTAdesign™ liquid chromatography systems. For practical reasons, the user documentation for ÄKTAcrossflow also includes the user reference manuals for the UNICORN general liquid chromatography version (the examples in the UNICORN User Reference Manual are based on an ÄKTAexplorer 100 system operating with the E100F400 strategy).

2.4.2 Software modules

The software consists of four integrated modules:

- UNICORN Manager for file handling and administration, e.g. definition of systems and user profile etc.
- Method Editor to create and edit methods for pre-programmed system control.
- System Control to monitor processes on line.
- Evaluation to evaluate and present stored results. It also includes the Evaluation Wizard specifically designed for ÄKTAcrossflow result files.

2.4.3 Common interface

UNICORN provides a common control platform and one common user interface for all scales of operation. In addition there is the same familiar interface for both chromatography and membrane systems which can be controlled and monitored from your office desk, with easy-to-use software wizards.

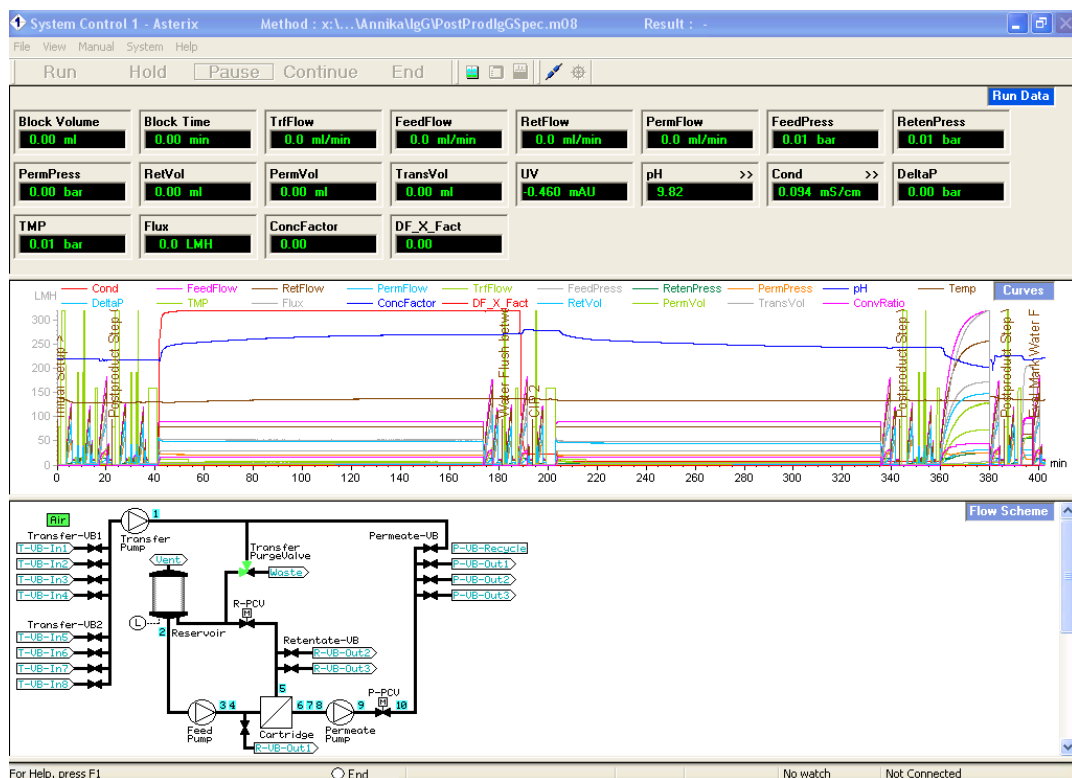


Fig 2-8. Common interface for chromatography and membrane operations.

2.4.4 Special features

The scouting feature gives automatic support to process development and optimization. Method wizards and pre-programmed cleaning methods provide a high degree of efficiency in scale-up and production processes, and simplify purification tasks. The ability to generate customized reports saves time, and the software is supplied with comprehensive documentation that helps fulfil regulatory requirements. UNICORN fully conforms to all applicable regulations including 21 CFR Part 11.

Complete documentation and protection

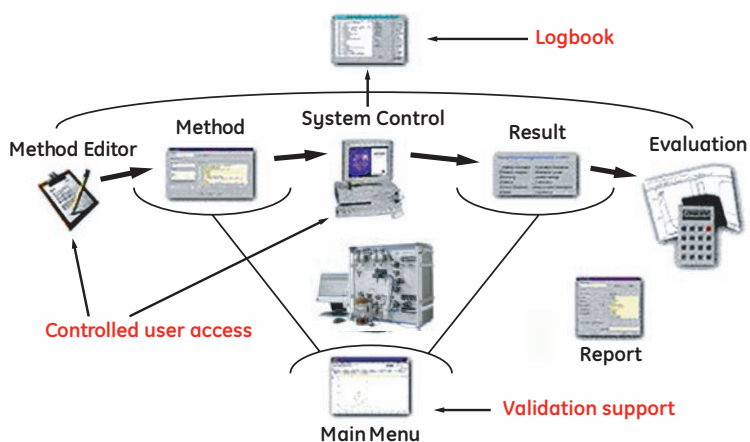


Fig 2-9. Complete documentation and protection.

2.4.5 Control modes

ÄKTAcrossflow system with UNICORN software supports the process control modes commonly used in ultrafiltration/diafiltration and microfiltration applications, such as TMP control and flux control. These control modes can be combined with selectable feed pump instructions such as, feed flow rate, feed pressure, ΔP , retentate flow rate or shear rate. UNICORN also reports real-time process parameters such as retentate volume, concentration factor, diafiltration exchange factor (total buffer used /retentate volume) and accumulated permeate volume.

2.4.6 TMP control

TMP control is usually used in ultrafiltration where the system forces the retentate through the relatively small pores of the membrane. The TMP control mode is used at a constant feed flow, a constant retentate flow or a constant ΔP .

TMP control mode	Control element:		
	Feed pump	Permeate pump	R-PCV
TMP control with constant Feed flowrate	$Q_F > 0$	Offset	TMP
TMP control with constant Retentate flowrate	$Q_R > 0$	Offset	TMP
TMP control with constant ΔP	$P_F - P_R > 0$	Offset	TMP

Table 2-2. TMP Control mode.

The TMP is mainly controlled by the retentate control valve (R-PVC). In the TMP control mode the software adjusts the retentate valve and permeate pump to maintain a constant TMP. In TMP control mode the offset is 0.2 bar as default, and is used to avoid low or negative pressure on the permeate side, which would affect the permeate pump's function as a flow meter.

2.4.7 Flux control mode

Flux control is usually used in microfiltration where the system limits the permeate flow through the relatively large pores of the membrane. The control mode is used at a constant feed flow, a constant retentate flow, a constant shear rate or a constant ΔP . In this mode the TMP value is a function of the permeate flux.

Flux control mode: Flux Control	Control Element:		
	Feed Pump	Permeate Pump	R-PCV
Flux control with constant Feed flowrate	$Q_F > 0$	Flux > 0	Offset, Unrestricted for $P_p > \text{Offset}$
Flux control with constant Retentate flowrate	$Q_R > 0$	Flux > 0	Offset, Unrestricted for $P_p > \text{Offset}$
Flux control with constant Shear rate	shear rate > 0	Flux > 0	Offset, Unrestricted for $P_p > \text{Offset}$
Flux control with constant ΔP	$P_F - P_R > 0$	Flux > 0	Offset, Unrestricted for $P_p > \text{Offset}$

Table 2-3. Flux control mode.

During flux control it is common that the feed pressure is so low that the permeate pressure drops below zero. If the permeate pressure is below 0.2 bar, the software will adjust the R-PCV to increase the retentate pressure. When the permeate pressure is above 0.2 bar the permeate pump can start. A constant ramping during 60 seconds from flux zero to the set flux value is then performed.

2.5 Programming a UNICORN method

2.5.1 Blocks

The text pane in the Method Editor of UNICORN displays the method as a list of text instructions (Fig. 2-10). The instructions are usually organized in blocks, which define a specific function (e.g. 'load a sample', or 'concentrate a sample'). Blocks are indicated by blue square symbols. A block may contain other blocks or individual instructions. The blocks can be expanded to show the instructions within the block.

2.5.2 Base

Every method must start with a base instruction, defining the base for calculating breakpoints. Different blocks can use different bases. In ÄKTAcrossflow the default method base refers to "column volume" and thus needs to be changed to one of the following:

- Volume (the unit depends on the scale defined in the system strategy)
- Time (minutes)
- SameAsMain (all blocks will inherit the base defined in the main block)

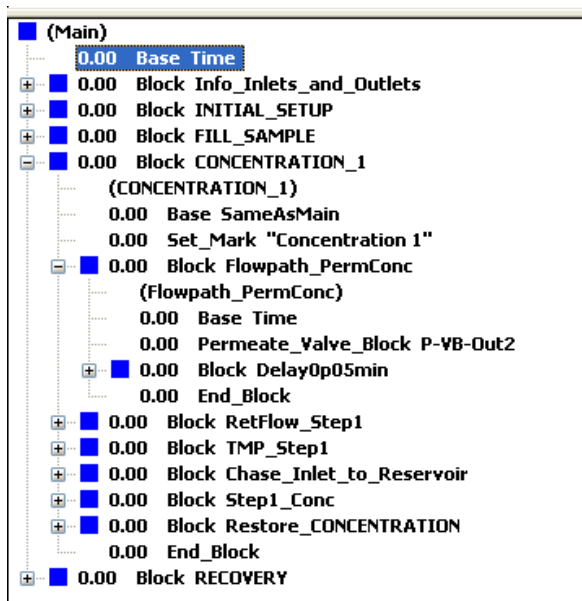


Fig 2-10. UNICORN text pane in the Method Editor

2.5.3 Calls

To execute the instructions contained within a block in a method, the block must be called by the program. When a block is called the instructions in the block are executed in the order that they are written until the block is finished or the **End_Block** instruction is executed.

There are two types of calls:

- Unconditional calls, which are made with a Block instruction
- Conditional calls which are made with a Watch instruction. This makes it possible to call a specified block or instruction when a particular monitor signal meets a given condition.

2.5.4 Watch and Hold_Until

The breakpoint when the Watch instruction is issued determines when the Watch begins. A Watch remains active until the condition is met or a new Watch instruction is issued for the same monitor. The Watch is cancelled automatically when the condition is met. A Watch can also be turned off with the **WATCH_OFF** instruction.

The **Hold_Until** instruction is a special kind of Watch instruction. The method is put on hold until a specific condition is met (signal, test or value) or the time-out is reached. Thereafter the remaining instructions in the method are executed.

2.5.5 Block pane

The organization of blocks in the method is shown graphically in the Block Pane of the Method Editor (fig. Fig. 2-11). Each block is represented by a gray bar with the block name and the length of the block. The line is shifted down to indicate calls to other blocks.

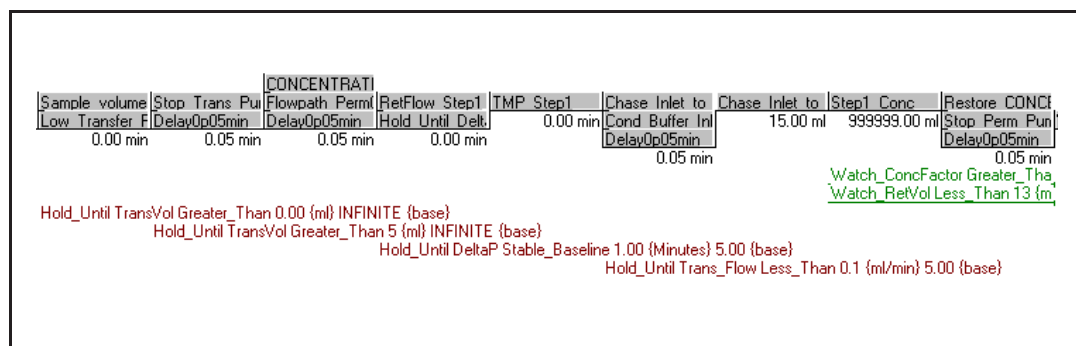


Fig 2-11. A Block pane in the Method Editor.

2.5.6 Run Set up

Run Set up in the Method Editor is a dialog box with a number of tabs that define the method properties (Fig. Fig. 2-12).

Start Protocol		Questions		Result Name	
Variables	Scouting	Notes	Gradient	Columns	Reference Curves
			Evaluation Procedures		
			Method Information		
Block	Variable	Value	Range		
Start_Settings_System	Ret_Vol {ml}	13.00	0.00 - 5000.00		
	Feed_Press_Alarm {bar}	5.20	0.00 - 5.20		
	TMP_Alarm {bar}	5.20	0.00 - 5.20		
Start_Settings_FS	Filter_Area {cm ² }	50.0	1.0 - 1000.0		
Sample_volume	Sample_Vol_High {ml}	0.00	0.00 - 80000.00		
	Sample_Volume {ml}	5.00	0.00 - 80000.00		
RetFlow_Step1	Ret_Flow_Step1 {ml/min}	25.0	0.0 - 600.0		
Hold_Until_DeltaP	DeltaP {bar}	0.05	0.00 - 5.20		
TMP_Step1	TMP_Step_1 {bar}	2.00	0.00 - 5.20		
Step1_Conc	Conc_Factor_Step1Cont	50.00	0.00 - 50.00		
	Ret_vol_Step1Cont {ml}	13.00	0.00 - 80000.00		
RetFlow_ProdRec	Ret_Flow_ProdRec {ml/min}	25.0	0.0 - 600.0		
Ret_Flow	Ret_Flow {ml/min}	40.0	0.0 - 600.0		

☐ Show details
☐ Show unused variables
☒ Display tooltip for extended variable cells

[Edit Variable...](#)
[Help](#)

Fig 2-12. Run Set up dialog box in the Method Editor.

2.6 Work flow

The work flow for ÄKTAcrossflow can be divided into three distinct stages:

- Create a method
- Run the method
- Evaluate the results

2.6.1 Creating a new method

There are two ways to create a new method:

- Using the Method Wizard, where customized methods for most methods are pre-programmed, and the user sets appropriate values for the method variables
- Using the Text Instructions editor in the Method Editor module, where the user can choose more advanced editing facilities

2.6.2 Method Wizard

Method wizards support all typical ultrafiltration and microfiltration product processing operations. With the method wizard, filtration methods can be easily and rapidly programmed. It uses pre-optimized and verified methods, and no programming skills are needed. The method wizard covers system functional tests and all the steps in a typical filtration process. It is possible to rinse new filters, CIP used filters and test water flux to check filter quality and status before and after each run. Data for a given filter can be gathered over multiple cycles in order to check its membrane flux recovery. A system sanitization method is also provided.

Wizard methods for flat sheets	Wizard methods for hollow fibers		
Ultrafiltration	Cell processing		
Proteins	Cell harvest	Cell clarification	Lysate clarification
1. Concentration (reduce volume)	1. Concentration (reduce volume)	1. Concentration (reduce volume)	1. Concentration (reduce volume)
2. Diafiltration (exchange buffer)	2. Washing (promote contaminant passage)	2. Washing (promote product passage)	2. Washing (promote product passage)
3. Recover product (retentate)	3. Recover product (retentate)	3. Recover product (permeate)	3. Recover product (permeate)

Table 2-4. There are Method Wizards for all typical product processing operations.

2.6.3 Choosing a filter type

Before starting the method, the filter type is defined in UNICORN manager, which determines the choice of cell processing or ultrafiltration in the method wizard. To get the Method Wizard for hollow fibers, "Hollow Fiber" must be selected as the filter component in the System Setup Component dialog. To get the Method Wizard for flat sheets, "Flat sheet" is selected.

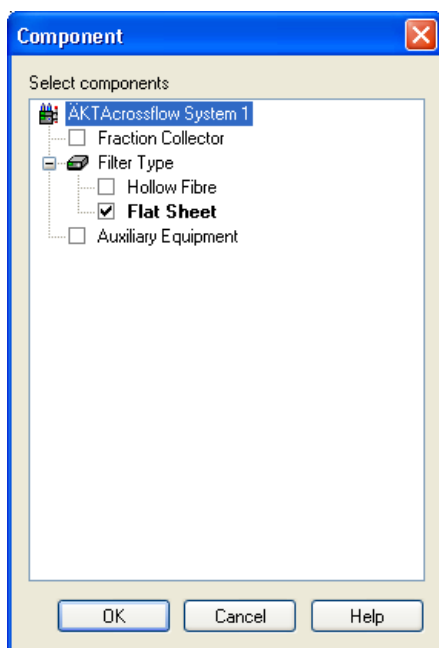


Fig 2-13. During system preparation the filter type is chosen in UNICORN manager.

2.6.4 Creating a method

Creating a method is easy and straightforward by choosing the method from a number of pre-defined basic settings. For product steps, up to three concentration or diafiltration/wash steps can be selected. It is also possible to run in fed batch or tank batch mode depending on the initial processing volume.

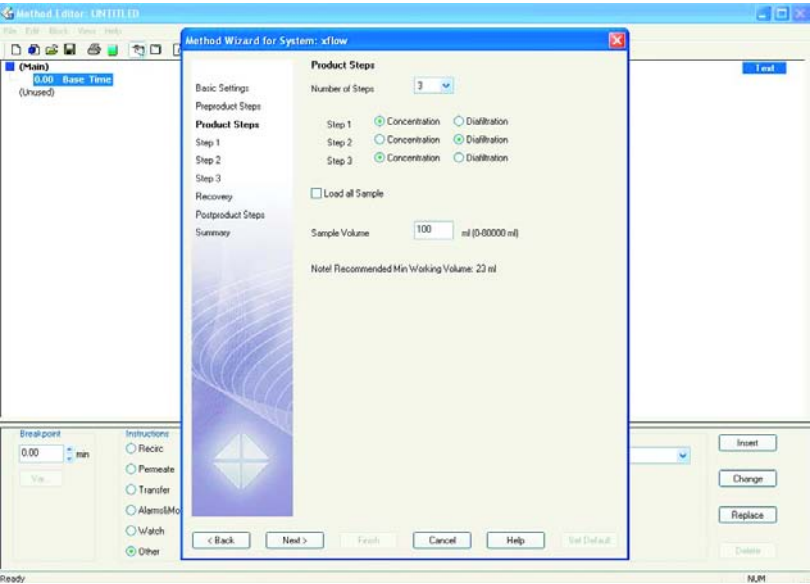


Fig 2-14. Product step selection in the method wizard.

It is also possible to choose the pre-product and post-product steps to be included in the method. Note: the methods are slightly different for hollow fibers cartridges and flat sheet cassettes.

Pre-product steps	Post-product steps
Rinsing	Flush
Filter CIP	Filter CIP
Water flush	Water flush
Water Flux test	Water Flux test
Buffer conditioning	Filter storage solution

Table 2-5. Pre-product and Post-product steps contained in the Method Wizard.

2.6.5 Process optimization

A special option in the Method Wizard is the process optimization method where specific recirculation conditions are selected for scouting a series of TMP values to find the optimal TMP for the application being run. This method is automatically linked to the evaluation module where the results can be analyzed and presented.

2.6.6 Evaluation module

The evaluation module eliminates manual transfer of data to spread sheets. The module allows flexible and direct presentation of optimization results with five different evaluation operations.

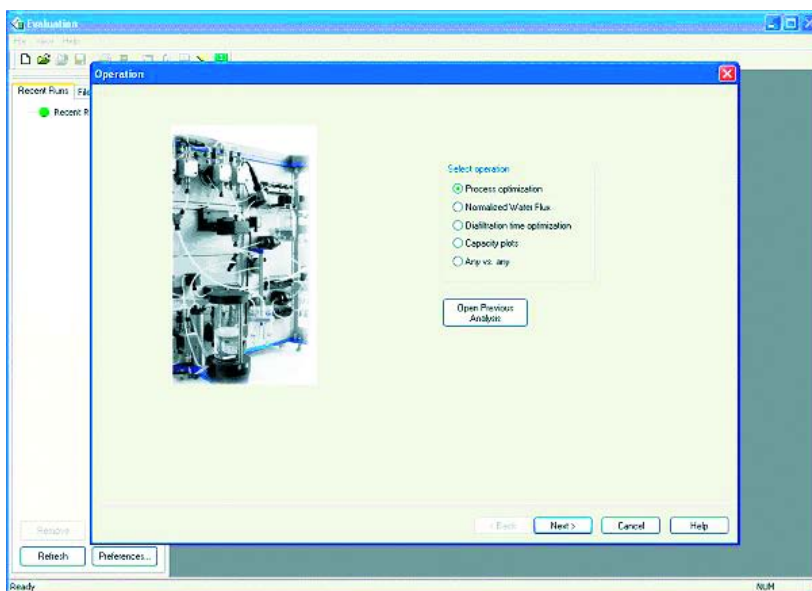


Fig 2-15. Operations available in the evaluation module.

The operations include process optimization, normalized water flux, diafiltration time optimization, capacity plots and 'any vs. any' plots.

Process optimization is used to analyze process characterization experiments where a series of set points are tested.

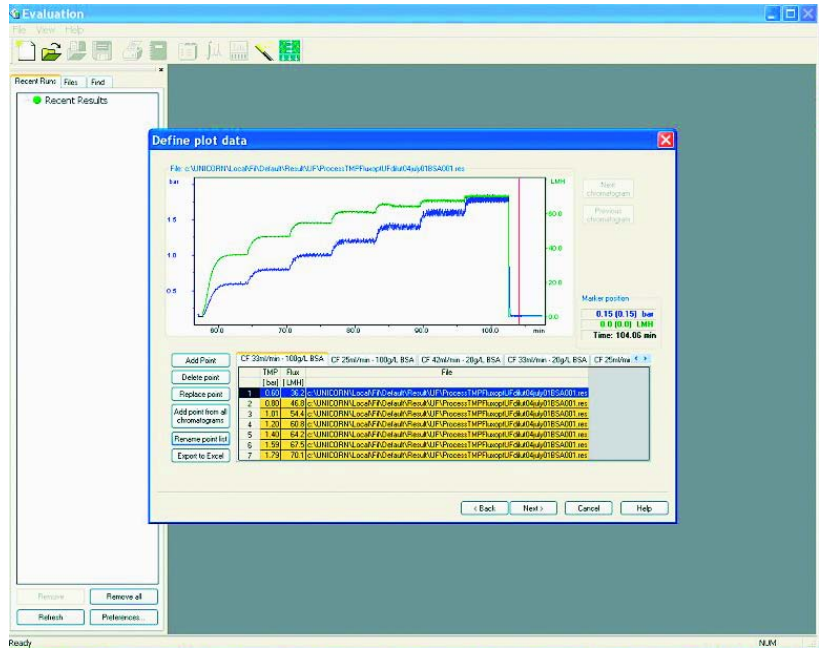


Fig 2-16. Raw data from process optimization for BSA concentration/diafiltration using TMP scouting with 100g/l and 20g/l BSA solutions.

The most common experiments are TMP excursions at different retentate flow rates and protein concentrations. Process optimization makes a new plot from user identified points along original data curves, for example flux vs. TMP. Process optimization also allows multiple plots to be overlaid at different retentate flow rates or protein concentrations. This capability can be used for any process parameter.

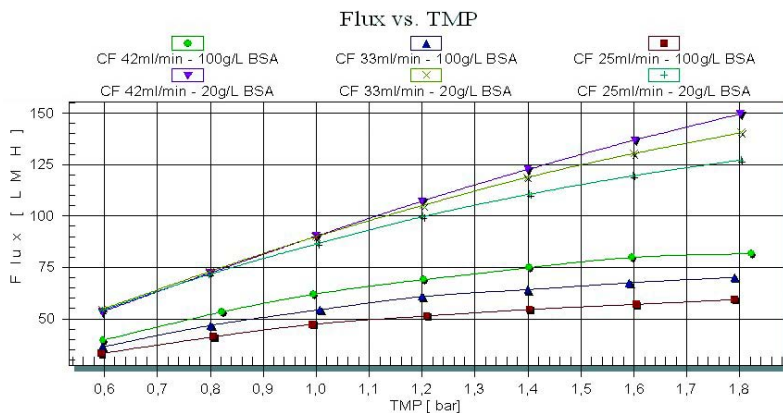


Fig 2-17. Optimization curves generated in the evaluation module from the raw data of Fig. 2-16.

Normalized water flux is used to monitor membrane quality over several cleaning cycles. This ensures that the cleaning process is still effective and also helps to determine the lifetime of the filter.

For a given ultrafiltration process diafiltration time optimization allows the user to identify the factor of volume concentration where the least time is required to complete the diafiltration.

The analysis of experimental results in cell processing often includes plotting process parameters versus the membrane capacity. Capacity plots allow the user to plot any process parameter, including a system-external result such as activity assay results, versus the accumulating permeate volume normalized to the surface area (capacity).

The any vs. any evaluation operation is used to analyze results from routine concentration, diafiltration and cell processing runs. It allows any process parameter captured as a curve in a given result file to be plotted on either the X-axis or the Y-axis.

2.7 Comprehensive report generation

UNICORN software provides a flexible environment for presenting data and information. It includes configurable report formats for easy report generation, and has a simple click and print report interface. All information about a run can be included and reports can be printed automatically after a run. It is also possible to export data to Microsoft® Excel® for further analysis if required.

2.8 Security

UNICORN software has an extensive data analysis capability, while maintaining data security.

Feature	Function
Access security	Only authorized users can access UNICORN. Each user is assigned an access level, which defines the functions that the user is permitted to use.
Data security	Result files from an ongoing run can be saved automatically at preset intervals to minimize data loss if the system fails. The results are saved locally if the network communication fails.
Electronic signatures	Method and result files can be signed electronically for enhanced security and accountability.

Table 2-6. Main security functions in UNICORN for ÄKTAcrossflow.

Original data cannot be modified or deleted and an evaluation audit trail shows all access and operations performed. All batch details are included in a single result file for easy back up, and all method details are embedded in a result file. In addition, the design of the evaluation module allows the automation of repetitive tasks.

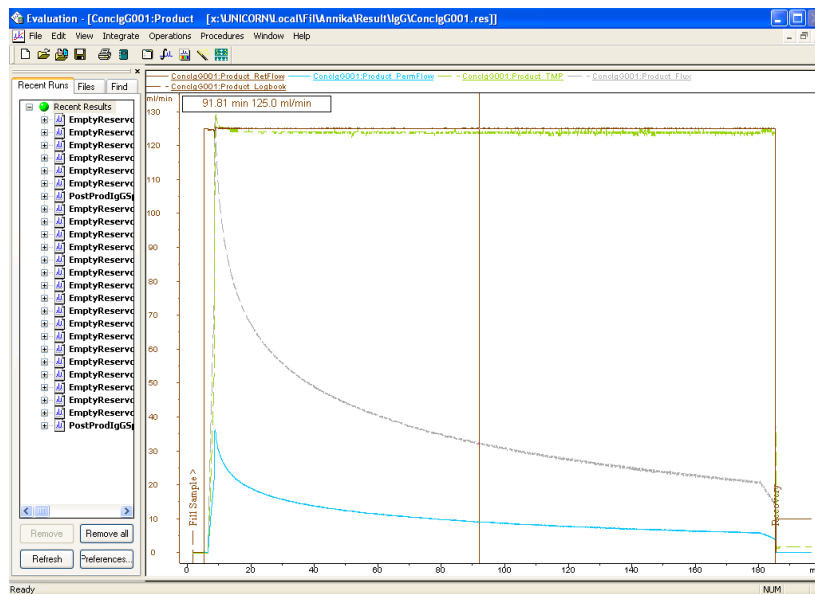


Fig 2-18. Result file.

Detailed descriptions of creating and running a method, and of evaluation the results can be found in ÄKTAcrossflow User Manual and the UNICORN 5.0 Evaluation for Cross Flow Filtration User Reference Manual.

3 Cross flow filtration process considerations

3.1 *Factors influencing product yield*

The amount of product that can be recovered from a process step represents the product yield. Increasing yield in laboratory processes ensures maximum product for testing, efficient use of lab resources, and accurate projections for scaling to pilot equipment.

3.1.1 General considerations

The system tubing design can affect product yield if it impedes recovery of the liquid. For example, every CFF system has a hold up volume (tubing volume), but well-designed systems minimize this volume, enabling maximum product recovery. Poorly designed systems include long tubing runs, poor tank drainage, and other non-recoverable volume such as poorly positioned drain valves. Two methods can be used to recover most of the hold-up volume from ÄKTAcrossflow system. One method maximizes product yield at the expense of concentration, and the other enables the highest concentration to be achieved at the expense of some yield.

3.1.2 Measuring yield

When yield becomes important, the appropriate process streams should be sampled before and after filtration. By sampling the permeate stream, information can be obtained on the types of non-target proteins, lipids, or unwanted components that are being passed through the membrane. ÄKTAcrossflow includes a UV sensor in the permeate line to measure the passage of protein through the membrane.

3.2 *Specific actions that increase yield*

The key factors that influence yield are the following:

- Membrane selection
- Non-recoverable hold-up volume
- Denaturation: shear, temperature, and enzymatic action
- Concentration and gel layer formation

3.2.1 **Membrane selection**

For ultrafiltration with cassettes, membranes influence ultrafiltration yields in two ways: Selectivity and protein binding. If a membrane is selected with pores too large to retain the protein being concentrated, some of the target protein passes through the membrane and is not recovered, decreasing yield. Protein binding usually becomes an issue when attempting to separate extremely small amounts of protein. In this case, binding of the protein to the membrane can show up as unexpected yield losses. KwickStart polyethersulfone membrane exhibits low protein binding and minimizes this effect.

For cell harvesting with microfiltration cartridges, membrane selection plays a less important role in yield results. Cells are relatively large compared to the membrane pores. So even selecting a microfilter with very large pores will still retain all of the cells and particle components. For lysate clarification, where the goal is to recover a protein while holding back cell debris, membrane selection is critical in allowing the target protein to pass.

Membrane selectivity

Membrane selectivity is defined as a membrane's ability to retain 100 percent of a single species.

Ultrafiltration filters have a broad pore size distribution and are therefore not highly selective. To achieve the best possible retention with a typical ultrafiltration filter, a NMWC that is 3 to 5 times less than the target molecule weight should be evaluated for performance.

In microfiltration, membrane selectivity is not as critical. For example, when separating an antibody from a cell culture, pore size distribution is not a key factor. A membrane with a distribution of larger pores will provide good yield but the permeate may be slightly turbid and require a polishing filtration step. If an excessively small pore size is chosen, not all of the antibody will pass through the membrane decreasing the yield.

The following guidelines represent a good starting point for microfiltration membrane selection:

- Yeast and bacteria cell harvest-0.1 μm pore size microfiltration
- Lysate clarification-microfiltration pore size about 10x larger than the target protein
- Mammalian cell clarification-0.2 to 0.65 μm pore size microfiltration

Membrane protein binding

The level of protein binding depends upon the membrane material and the protein characteristics, and increases with increasing hydrophobicity (Table 3-1). Normally, in terms of yield, protein binding remains insignificant at the laboratory scale, but for tight ultrafiltration membranes it can be an indicator of a propensity towards membrane fouling.

Membrane type	BSA ($\mu\text{g}/\text{cm}^2$)	Lysozyme ($\mu\text{g}/\text{cm}^2$)
10 kD select	1.8	4.5
10 kD	1.6	4.2
30 kD	2.4	5.2
50 kD	2.4	5.1
100 kD	9.6	5.2

Table 3-1. Typical dynamic protein binding capacities for membrane¹.

¹ Data from Validation Guide for Amersham Biosciences Membrane Cassettes, document number 18-1171-70 AA, published by GE Healthcare. The dynamic protein-binding test involved installing membrane into a stirred cell, pre-wetting the installed membrane with buffer, and then passing the protein solution through the membrane. Following exposure to the protein solution, membrane discs were washed three times to remove unbounded proteins. Proteins that remained on the membrane were analyzed using a BCA kit. Membrane dynamic-protein-binding capacity is reported in $\mu\text{g}/\text{cm}^2$.

Cartridge selection

Cartridge selection is influenced by process objectives and operating variables. This is summarized in Table 3-2 .

Process objective	Cartridge selection
Cell concentration Cell protein separation	Use microfiltration or open ultrafiltration cartridges for bacterial removal and cell concentration. Select membrane pore size based on the specific application.
Virus removal Protein concentration Desalting	Use ultrafiltration cartridges for molecular-scale applications such as desalting and protein concentration.
Solution variables	Cartridge selection
Solids loading Viscosity Shear sensitivity	High solids loading and high viscosity fluids work best with larger hollow fibers and shorter lengths. With fluids that are not shear sensitive, small diameter fibers can be used.
Other variables	Cartridge selection
Time constraints	Increased membrane area and larger housing size shorten production time.
Pump constraints	Larger diameter (large surface area cartridges with many large fibers require pumps with high flow rate capacities).
Heat sterilization	Choose autoclavable or steam-in-place models.

Table 3-2. The influence of process objectives and operating variables in selecting a hollow fiber cartridge for microfiltration.

3.2.2 Recovery

Yield decreases as the quantity of process fluid that cannot be recovered from a system increases. ÄKTAcrossflow software Method Wizard supports two methods for recovery of the product:

- No recovery: An option to select if the retentate volume is to be drained manually.
- Recovery: The retentate is first emptied until the reservoir volume is zero. Then a maximum of two flushes are performed to flush the retentate side of product.

Minimum working volume

The minimum working volume represents the amount of feed/retentate fluid required to operate the system at the desired cross flow rate without drawing air into the feed pump. The minimum working volume is determined by the design of the system (retentate tubing volume, reservoir bottom design), the device retentate hold-up volume, and the crossflow rate. It is important to consider the minimum working volume of a system in the design of a CFF process; in particular, to confirm that the final target retentate volume is not less than the system's minimum working volume. For further details please refer to ÅKTAcrossflow Instrument Handbook.

3.2.3 Denaturation: shear, temperature, and enzymatic action

Excessive shear, temperature, and enzymatic action can denature the product and lower yield.

Shear

The shear sensitivity of a biomolecule generally increases with molecular size. Most proteins are relatively resistant to shear denaturation. If the shear sensitivity of the protein is not known, trials should be carried out to determine the relationship between process conditions and yield losses due to shear. As a quick feasibility study, a protein solution can be circulated across the feed retentate path and the bioactivity of the protein analyzed to relate protein activity to process time on a number of pump passes. Where feasible, low pressures and low pump speeds should be used to minimize shear in the flow path. When using hollow fiber filters, cross flow rates are often expressed in terms of shear rate. This convention makes it possible to scale up or down between cartridges. By using a shear reference chart, it is possible to approximate the flow rate that will yield the same shear at the new scale. The formula below can be used to calculate flow rates and shear rates for hollow fiber units:

$$\gamma = 4q \div \pi R^3$$

Where:

- γ = shear rate, sec^{-1}
- q = flow rate through the fiber lumen, $\text{cm}^3/\text{sec}/\text{fiber}$
- R = fiber radius, cm

Calculation of shear for cassettes is more complicated because of the influence of screens and is beyond the scope of this handbook.

Temperature

For heat sensitive proteins the process solution temperature can be modified in a number of ways during processing:

- Precondition the ÄKTAcrossflow system with cooled buffer before starting
- Lower the protein temperature before beginning the filtration process
- Use chilled buffer during diafiltration
- Use low pressure and low pump speed to prevent heat generation in the flow path
- Use low volume to filter surface area ratios to shorten process time
- Place the system in a cold room

Enzymatic action

Enzymes released during cell culture tend to concentrate in the concentration and gel layer. During concentration, lower molecular weight proteins pass through the membrane, and the concentration of the target protein increases. The enzymes may digest the proteins of interest which remain in high concentration in the feed/retentate circulation loop. Catalysts for enzymatic activity include heat, metal ions, and pH.

Enzymatic activity can be minimized by:

- Minimizing process time
- Minimizing process volume to surface area ratio
- Reducing temperature
- Adjusting temperature and ionic strength

3.2.4 Concentration gradient layer

During filtration, the solvent nearest the surface of the membrane flows through the membrane into the permeate. As the solvent flows away from the surface of the membrane, the solutes or particles near the surface become more concentrated (Fig. 3-1). This region of increased concentration is called the concentration gradient layer. The concentration gradient layer reduces the flux compared to clean water flux. The concentration gradient layer cannot be eliminated under production conditions, but it can be controlled to some degree. Decreasing TMP can lower the concentration gradient layer and its effects on flux. Increasing the cross flow rate produces a sweeping effect that helps to redistribute concentrated solutes back into the bulk feed stream and maintain flux.

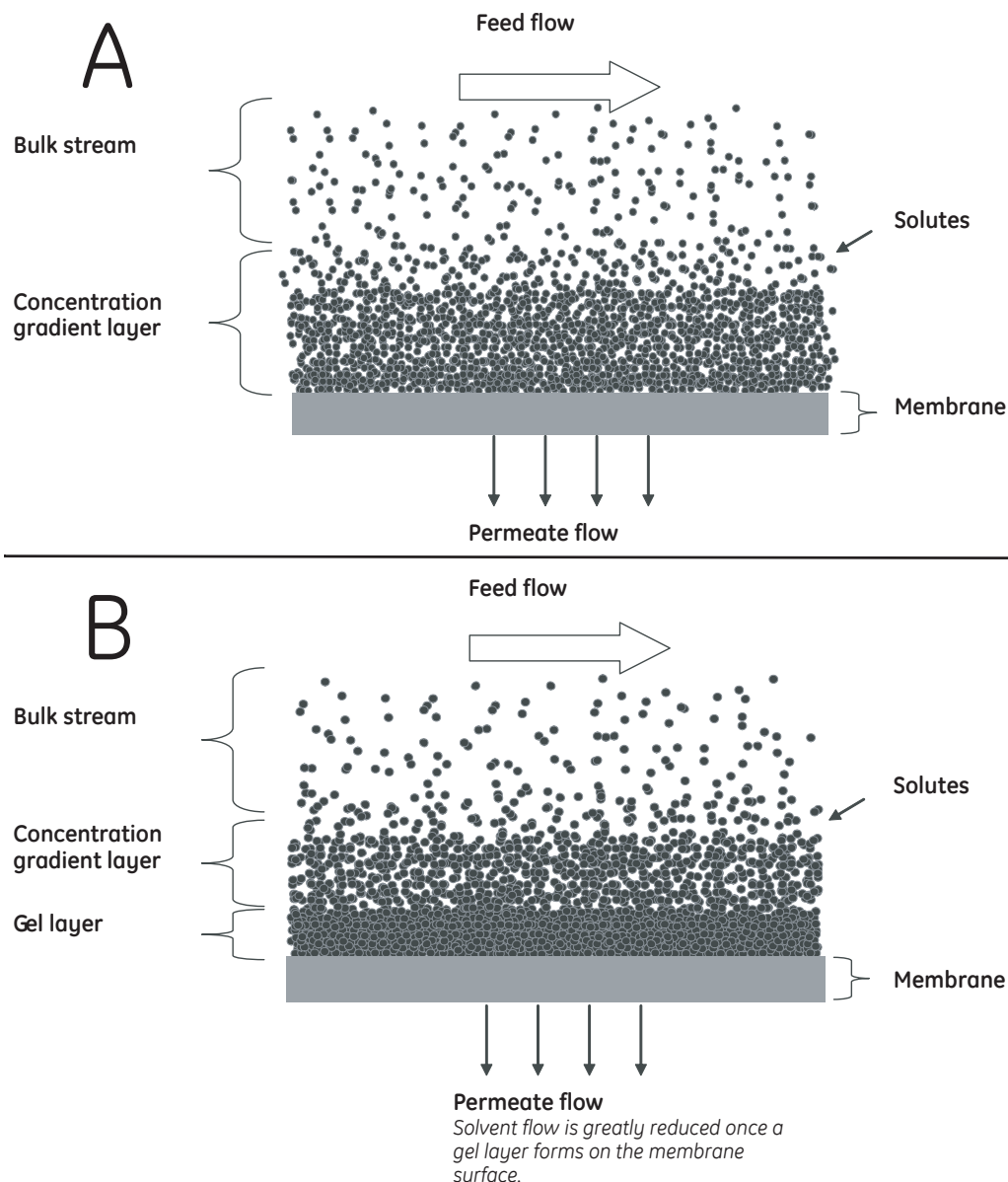


Fig 3-1. (A) Concentration gradient layer forms on the membrane surface during processing, and (B) gel layer and concentration gradient layer formed on a membrane surface.

3.2.5 Gel layer

A gel layer is a concentration gradient layer that has reached its highest value. In a gel layer, hydro colloids formed from concentrated proteins become packed so tightly against the membrane surface that they form a viscous or gelatinous layer. The gel layer has a considerable effect on the filtration process, influencing both filter efficiency and selectivity. To control the filtration process, steps must be taken to minimize the formation of a gel layer. (Fig. 3-1).

The following operating conditions contribute to gel layer formation:

- Excessive TMP
- Low cross flow rate
- High feed concentration
- Incorrect ionic condition of the feed

During the optimization of a CFF process with various TMPs, the point just before the formation of a gel layer is identified. At the optimum TMP and cross flow rate, the highest flux rate is achieved without forming a gel layer that will diminish process control and flux rate. In the case of protein concentration, where the product of interest is retained in the retentate, a gel layer can prevent the washing out of contaminants. The result is a reduction in purity and product quality.

3.2.6 Summary of concentration gradient and gel layer formation

In summary, three components resist the transfer of solvent through the membrane during concentration: the membrane, concentration gradient layer, and gel layer. With pure water, only the membrane resists the transfer and there is no concentration gradient or gel layer. When using process fluid, the membrane and the concentration gradient layer resist the transfer of solvent through the membrane. When a gel layer forms due to the incorrect operating conditions listed above, all three components (membrane, concentration gradient layer, and gel layer) resist the transfer of solvent, with the gel layer providing most resistance.

3.3 *Flushing product out with buffer*

Flushing product out of the filtration system with buffer enables the highest yield to be obtained. In this technique, the product should be slightly over concentrated, collected from the system, and a small volume of buffer or permeate added into the system. The added volume flushes out the residual product from the feed retentate loop.

ÄKTAcrossflow system should be programmed to perform the following steps:

- 1 As the CFF process nears completion, decrease the pump speed to minimize flow rate, vortexing in the feed tank, and the possibility for product foaming.
- 2 When the slightly over concentrated volume is reached, pump the concentrated product to the collection vessel.
- 3 Add an appropriate volume of buffer or permeate to the reservoir via the transfer pump. The buffer should be circulated for two to three minutes with the permeate valve closed to help bring the residual product into suspension.
- 4 Pump the buffer solution from the system into the collection vessel.

3.4 *Recovering product from the membrane surface*

Recovering product from the membrane surface enables the most highly concentrated product to be obtained. In this technique, product is recovered from the membrane surface without adding buffer or permeate to the system.

ÄKTAcrossflow system should be programmed to perform the following steps:

- 1 At the end of the process of harvesting cells or concentrating a protein, close the permeate valve or reduce the feed pressure to 0.3 bar (5 psi).
- 2 Reduce the cross flow rate to 1/10 of the recommended processing cross flow rate.
- 3 Circulate the remaining product for 15 minutes. This procedure will help recover product that has accumulated on the surface of the membrane.
- 4 Recover the product by pumping it from the system to a collection vessel.

3.5 *Product recovery and assay specificity*

Measuring recovery requires a reliable assay for the product. The assay must have a specificity for only the product of interest and not any degradation products that may be present. Mass balance estimates for recovery require feed samples before and after filtration, and permeate samples after filtration. An analysis of the permeate samples provides insight into the rate of product passage over the processing time.

The formula mass balance determination is as follows:

$$V_s C_s = V_r C_r + V_p C_p + V_h C_h$$

Where:

- V = volume
- C = concentration
- s = starting
- r = retentate
- p = permeate
- h = hold-up

3.6 Operating parameters

3.6.1 Flux versus TMP

In CFF, the key optimization parameter is the flux rate as a function of TMP. For a given cross flow rate, TMP controls flux at the beginning of a run. If a gel layer forms, increases in TMP will not result in increases in flux. Therefore increasing TMP will provide little performance gain. The optimal TMP range for efficient and economic operation is just before the gel layer starts influencing the flux (Fig. 3-2).

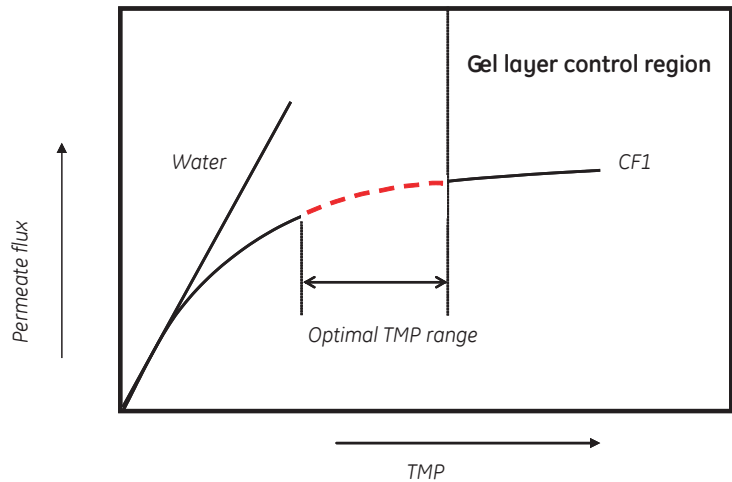


Fig 3-2. Optimal TMP range under a constant cross flow rate (CF1).

ÄKTAcrossflow system software includes a method that can perform an ultrafiltration optimization based on default values or values input by the operator. ÄKTAcrossflow system runs the optimization experiment and can display the results in table or graphic format. Data can be exported to Microsoft Excel for advanced analysis. See ÄKTAcrossflow System User Reference Manual for additional information.

3.6.2 TMP and crossflow

At a given TMP, increasing the cross flow rate helps reduce the concentration gradient layer and increases flux. Cross flow rates may be increased until process yield or product quality are adversely affected through, for example, shear effects. Optimization of a CFF process such as protein concentration must include an examination of the interaction of the two most important variables: cross flow and TMP. At the optimum combination of the highest cross flow and TMP, just before gel layer accumulation, the highest flux rate will be achieved (Fig. 3-3).

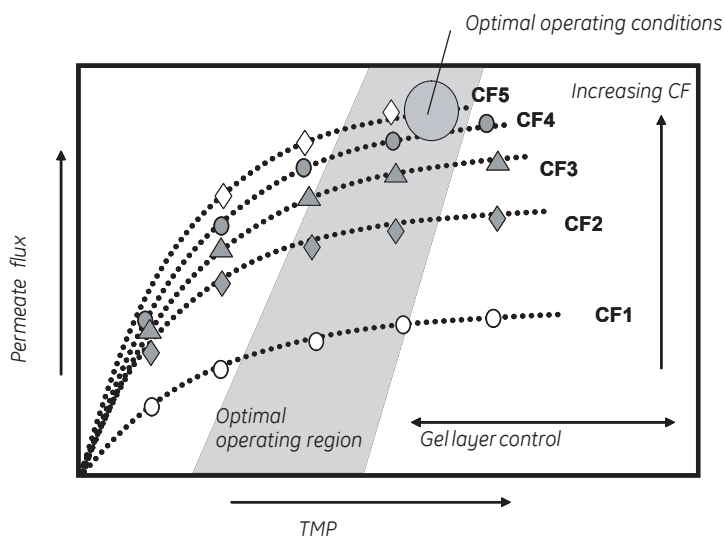


Fig 3-3. Relationship of TMP and flux at five cross flow rates.

3.7 *Scaling up parameters*

The ability to scale a process from the laboratory to manufacturing is a key factor in process development. Normally, the scale up sequence is completed in multiple steps: lab scale to pilot scale, and pilot scale to production scale. Reasonable scale-up increments are typically 5 to 20 times.

When scaling up, the following parameters should be kept constant:

- Ratio of filter area to feed volume
- Membrane
- Screen type if applicable
- Fiber or cassette path length
- Channel height (cassettes) or lumen size (hollow fiber cartridges)
- Cross flow rate
- TMP
- Temperature
- Feed concentration
- Process steps and sequence

The scale up pathway from ÄKTAcrossflow system to the UniFlux system and production systems is summarized in Table 3-3 .

GE Healthcare CFF systems and devices for scale-up		
Lab scale ÄKTAcrossflow system	Pilot scale UniFlux system	Production scale Engineered system
Kvick Start cassette Kvick Lab cassette	Kvick Flow-5 cassette Kvick Pilot cassette	Kvick Flow-25 cassette Kvick Process cassette
Start AXM, Start AXH	Pilot scale cartridges Process scale cartridges	Process scale cartridges

Table 3-3. Scale-up pathway for cartridges and cassettes.

3.8 Membrane fouling and cleaning procedures

Start AXM and Start AXH cartridges are designed for single use only. The recommended cleaning procedure for Kwick Start cassettes is summarized in Table 3-4 .

Cleaning agent	Cleaning conditions	Membrane type
1,5% Alconox® detergent	Contact time 60 minutes Temperature 40°C (104°F)	Polyethersulfone
0.1 to 0.5N NaOH	Contact time 60 minutes Temperature 40°C (104°F)	Polyethersulfone
200 to 300 ppm sodium hypochlorite in 0.1 to 0.5N NaOH	Contact time 60 minutes Temperature 20°C (68°F)	Polyethersulfone

Table 3-4. Recommended cleaning procedures for membrane cassettes.

3.9 Troubleshooting

The following chart can be used to troubleshoot fouling problems in CFF.

Symptom of fouling	Possible cause of fouling	Corrective action
During operation, slow gradual decrease of flux to about 90% of starting flux	Normal operation	None
During operation, moderate decrease of flux to about 75% or less of starting flux	Best pore size for application not being used	Reevaluate pore size and membrane area selection
	Insufficient cross flow rate, excessive gel layer formation	Increase cross flow rate
	TMP too high, excessive gel layer formation	Lower TMP, reduce permeate flow rate using permeate flow control
	Chemical incompatibility between cleaning agents and membrane, membrane damage	Check chemical compatibility between membrane and process fluid
Water flux after cleaning less than 60-80% of initial water flux	Insufficient cleaning	Increase cleaning temperature. Increase concentration of cleaning solution. Increase cleaning circulation time or rate. Use cleaning solution better able to solubilize contaminants.
	Chemical incompatibility between cleaning agents and membrane, membrane damaged	Replace cartridge or cassette.
Water flux less than 60% of water flux when the cartridge was new; data shows a gradual decrease over many runs.	Normal decline in operational efficiency	Replace cartridge or cassette.
Water flux less than 60% of water flux when the cartridge was new; data shows decrease was sudden compared to historical data.	Chemical incompatibility between cleaning agents and/or process fluids and membrane, membrane damaged	Replace cartridge or cassette.
	Insufficient cleaning	Increase cleaning temperature. Increase concentration of cleaning solution. Increase cleaning circulation time or rate. Use cleaning solution better able to solubilize contaminants.

Table 3-5. CFF troubleshooting chart.

4 Cell Processing

4.1 Cell harvesting

Cell harvesting is the process of separating cells from the fermentation broth in which the cells grow. After harvesting, the cells are mechanically disrupted and the protein of interest is separated from the cell debris by clarifying the lysate (Fig. 4-1). Hollow fiber microfiltration or higher NMWC ultrafiltration cartridges may be used effectively for cell harvesting.

4.2 Cell harvesting process

The concentration factor that can be achieved is based on the starting concentration which can, in the case of yeast cells, be as high as 70 to 80 percent by cell weight.

Typical concentration factors are:

- *E. coli* cells: 5x concentration
- Yeast cells: 2x concentration
- Mammalian cells: 10x to 20x concentration

4.2.1 Washing step

Cell harvesting usually includes a cell washing step to promote the transmission of broth components through the filter (Fig. 4-2). After washing, the ideal end product would consist of the concentrated cells suspended in the buffer used to wash the cells. However, in practice the harvested cells and buffer can contain varying levels of unwanted elements such as precipitated proteins, enzymes, and cell debris.

The washing process is commonly a constant volume diafiltration process, in which buffer is added to the cell suspension at the same rate as the permeate flow. Unlike centrifugal techniques where cells are packed in a dense cake or pellet, washing the cells in a buoyant state enables effective removal of contaminants. In ÄKTAcrossflow UNICORN software user interface, the diafiltration volume is used to set the washing parameters.

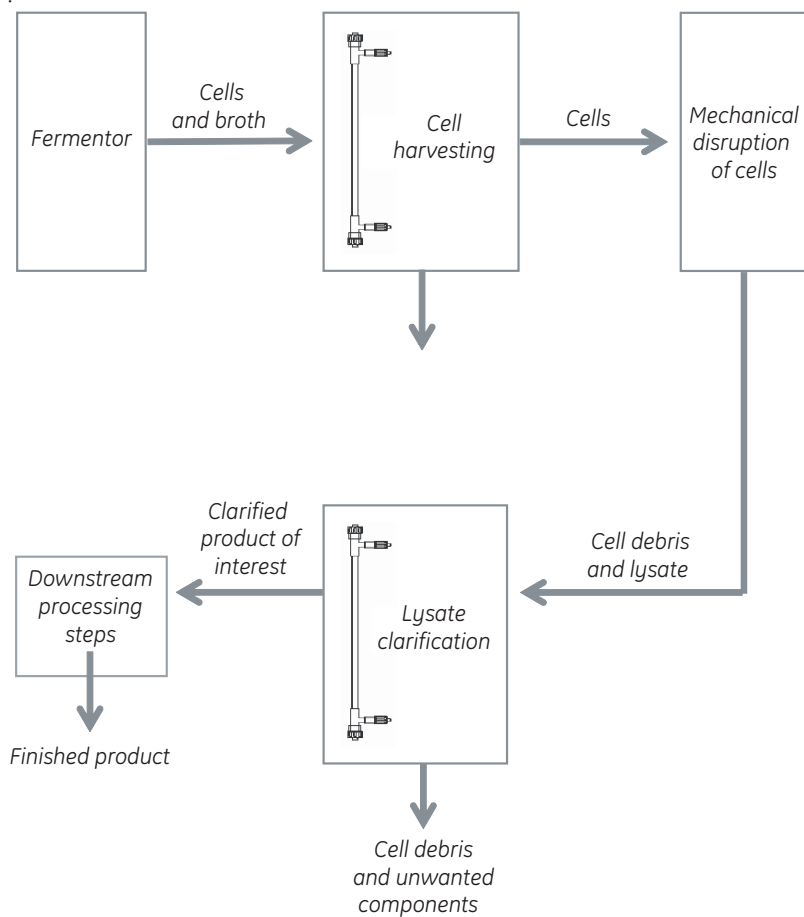


Fig 4-1. Cell harvesting step in a typical biopharmaceutical manufacturing process.

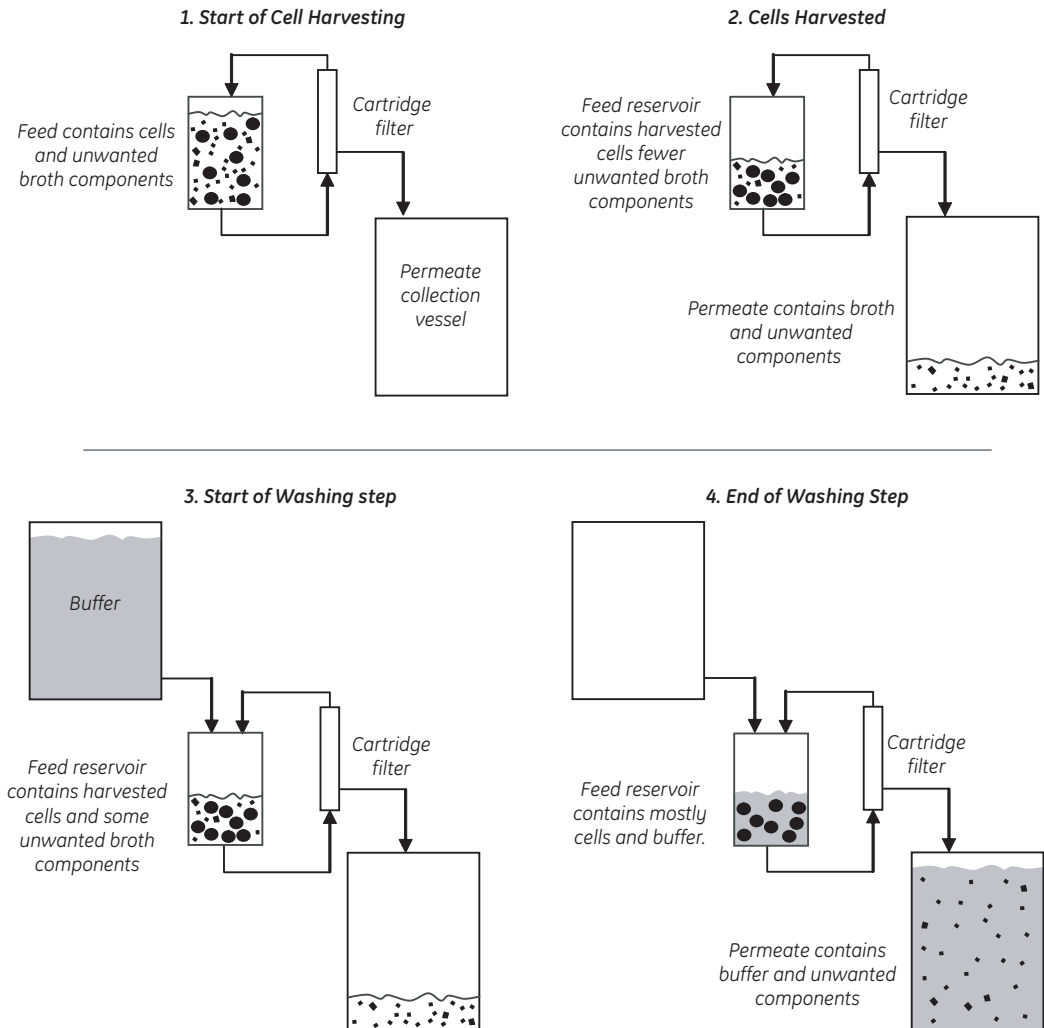


Fig 4-2. Cell harvesting often includes a washing step to help flush unwanted components from the fermentation broth.

Successful cell harvesting relies on knowledge of the product such as:

- Robustness of the cultured cells
- Starting volume and concentration of cells
- Desired finished concentration and volume
- Desired yield and quality (viability) of the cells

4.2.2 Typical steps in a cell harvesting method

The typical steps in a cell harvesting method are outlined in Table 4-1 .

Step	Comments
Rinsing	Rinse storage solution from filter
CIP	Circulate cleaning solution to clean system and filter
Water flush	Flush cleaning solution from system
Water flux test	Determines performance of filter before processing
Buffer conditioning	Conditions filter and system components before adding product to minimize adverse chemical reactions
Cell harvest (concentration)	Harvest cells
Cell washing (diafiltration)	Helps drive unwanted components through the membrane
Product recovery	Recovers cells from ÄKTAcrossflow system
Buffer flush	Flushes residual product from system without risking precipitation of components on the membrane or flow path
CIP	Recirculate cleaning solution
CIP (optional)	Recirculate cleaning solution
Water flush	Flush chemical cleaning solution from system
Water flux test	Determines the performance of filter after use and cleaning
Storage solution	Flushes the filter with storage solution to prevent bacterial growth in storage

Table 4-1. Typical steps in a cell harvesting method.

4.2.3 Membrane and cartridge selection

In cell harvesting, microfiltration membranes will easily retain all cells. The key to membrane selection is not based on retention, but on process optimization. For example, smaller pore size membranes often provide the highest permeate flux once the system is in a steady state (Fig. 4-3). The 500,000 NMWC ultrafiltration membrane is often the cartridge of choice for harvesting *E. coli*, even though it has a relative small pore size compared to the size of the cells.

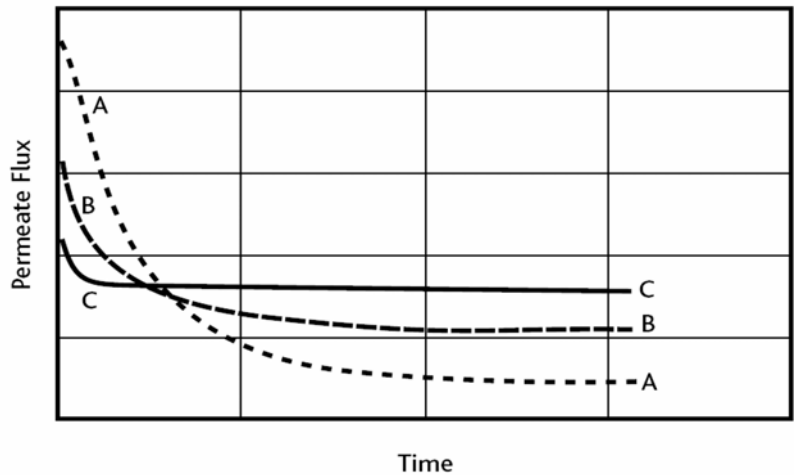


Fig 4-3. Flux of three membranes with all parameters held constant except pore size. Membrane A has a larger pore size than membrane B, which has a larger pore size than membrane C.

The 30-cm cartridge length allows low pressure drop for difficult separations using low TMP. The 60-cm hoop cartridges have a similar membrane area, but will require less circulation flow per unit area. Therefore, the 60-cm length is preferred for applications in which higher TMP does not adversely affect the separation (Table 4-2).

The inside diameters of the hollow fibers in Start AXM and Start AXH cartridges range from 0.5 to 1.0 mm. Larger diameter fibers should be used for solutions with high suspended solids, high cell densities, and high viscosity.

Type of cell	Suspended solids and viscosity	Path length (cm)	Lumen ID (mm)	Rating (μ)	Rating (NMWC)
E. coli	Moderate	30 or 60	1.0	0.1 - -	- 500,000 750,000
Yeast	High	30	1.0	0.1 0.2	750,000
Mammalian	Low	30 or 60	0.75 or 1.0	0.2 0.45 0.65	- - -
Blood cells	Moderate	30 or 60	0.75 or 1.0	0.2 0.45 0.65	- - -

Table 4-2. Recommended cartridges for cell harvesting.

Table 4-3 illustrates the relationship between process variables and cartridge selection.

Process variables	Selection considerations
Cell harvesting	Use microfiltration cartridges for cell harvesting. Select membrane pore size based on the specific application to achieve a stable flux rate.
Solution variables	Selection considerations
Cell concentration	Determine wet cell percent to anticipate the degree of concentration that may be used. A highly concentrated cell mass may seem efficient but may also result in high inlet pressures or lead to using the less efficient, short (30 cm) path length.
Solids loading	For whole cells, it is not uncommon to reach 70% wet cell weight while maintaining steady state conditions. However, lysates tend to need a lower solids level to promote passage of the target material. Start with solids in the 5 to 10% range and monitor transmission as well as TMP during the concentration phase.
Size of the target material	For separations with large target material (high selectivity) it may be best to avoid any concentration but rather perform a constant volume wash from the start. Using a more open membrane may require the use of short (30 cm) cartridges and permeate flow control. Use open UF membranes to clarify small proteins from either whole cell broths or lysate streams.
Shear sensitivity	If the feed stream is particularly shear sensitive and the recirculation flow rate is reduced, it may be necessary to lower the permeate flow rate (when using permeate flow control) to optimize throughput.
Volume	When scaling a process, cartridge housing diameter is increased in order to maintain constant volume to area ratio. When using a fixed 50 cm ² filter, estimate the flux rate so that the starting volume is suitable for the target process time.
Temperature	As temperature decreases, the filtration time often increases due to viscosity effects, and larger cartridges might be appropriate. For example, cold-room processing at 4°C can take twice as long as room-temperature processing.
Other variables	Selection considerations
Time constraints	Increased membrane area and larger housing size shorten production time.
Heat sterilization	Choose autoclavable or steam-in-place models for scale-up

Table 4-3. The influence of process variables and feed solution on the CFF process.

4.3 *Other product and processing factors*

There are some other factors that should be considered when defining a product and developing a process. A faster processing time may offer the benefit of less exposure of the product to shear forces, temperature increases, and enzymatic action.

4.4 *Preparation before use*

4.4.1 *Microfiltration cartridge*

Using a new microfiltration cartridge requires no rinsing. When using Start AXM and Start AXH hollow fiber cartridges, proceed to the water flux step.

ÄKTAcrossflow software automatically measures water flux, normalizes the results and presents and stores the data for analysis.

4.4.2 *Conditioning the system with buffer*

Before processing samples, it is recommended to precondition the system with a buffer similar in pH and ionic strength to that of the sample. Conditioning the system removes trapped air and minimizes unwanted chemical reactions between the sample and the wetted parts of the system.

- 1 Circulate 1 liter of buffer through the system with approximately 0.3 to 1 bar (5 to 15 psi) retentate pressure. Run until no bubbles appear in the permeate stream.
- 2 To ensure removal of trapped air, increase the retentate flow rate and run for several minutes until no bubbles appear in the retentate stream.
- 3 Circulate the buffer through the retentate and permeate at a feed pressure of 1.6 to 2.8 bar (25 to 40 psi) for four minutes to condition the system for pH and ionic stability.
- 4 Remove the buffer from the feed reservoir. Keep buffer in other parts of the system to prevent air from entering the system.

4.5 Operating parameters

4.5.1 Permeate flow control

In ultrafiltration applications, pressure (TMP) is applied to the filtration process to drive flux and efficiency. In most microfiltration applications such as cell harvesting, the flux is often so high, even at low TMPs, that the flux must be decreased to prevent premature fouling of the membrane. In ÄKTAcrossflow system, the permeate flow is controlled using the permeate pump. ÄKTAcrossflow software wizard enables the permeate flow rate to be set to control flux. Restricting permeate flow generates back pressure on the permeate side of the filter. The back pressure effectively lowers the TMP on the feed side of the filter, reducing the flux and pore fouling (Fig. 4-4).

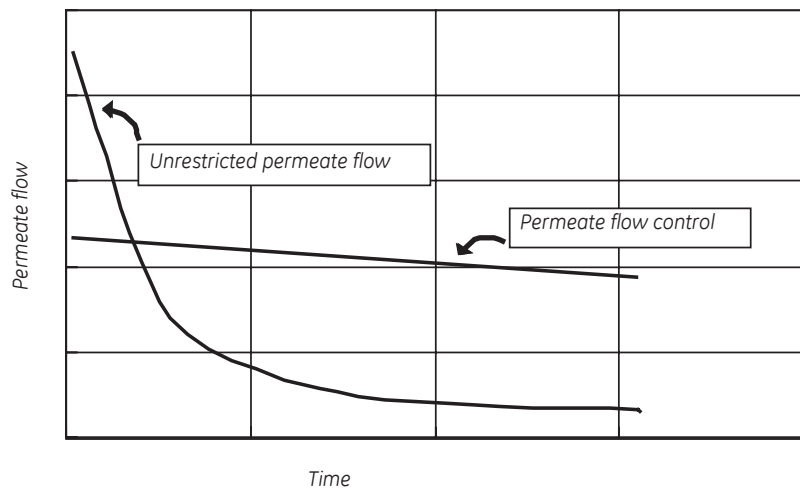


Fig 4-4. Using permeate flow control results in more stable flux.

Table 4-4 illustrates typical starting conditions for the harvesting of different cell types.

Bacterial cell harvest	Mammalian cell clarification	Virus particle concentration
10X concentration followed by 3X diafiltration	10X concentration followed by 3X diafiltration	5X concentration followed by 3X diafiltration
Average flux 25 l/mh with high cell density starting material and unrestricted permeate	Permeate flow control at 30 l/mh, no retentate back pressure	Low TMP and 6,000 sec ⁻¹ shear, 20-50 l/mh average productivity (calculated below at 30 l/mh)
This process description is for optimal recovery and washing of cells only	This process description is for removal of cells and optimal recovery of expressed target protein only	This process description is for purification of virus particles with gentle process conditions only

Table 4-4. Recommended starting point for developing process conditions for cell harvesting.

4.5.2 Recommendations for Start AXM and Start AXH cartridges

The general guidelines for using Start AXM and Start AXH cartridges in cell harvesting are as follows (typical operating flow rate):

- 8,000 to 16,000 sec⁻¹ shear rate for bacterial feed streams
- 2,000 to 4,000 sec⁻¹ for shear sensitive and high viscosity feed streams, including mammalian cells.

4.5.3 Process sequence

Flux (and protein passage) is dependent on the concentration of particles. With the high particle load typical of cell harvesting, low to moderate transmembrane pressures should be used, <1 bar (15 psi).

4.5.4 Process temperature

Room temperature is recommended, but only if process components are stable at this temperature; otherwise operate at 4 to 12 °C but with lower flux.

4.6 Cell harvesting conditions

When working with cells which may still be partially active, rapid methods may be important. This can be achieved by decreasing the process volume to

membrane area ratio. Using more membrane not only allows higher permeate flow rate, but it disperses any fouling agent over a broader membrane area. This results in a thinner fouling layer and consequently a higher average flux rate.

4.7 Cell clarification

Cell clarification refers to the separation of a target molecule from a cell culture. The cells are filtered and remain in the feed/retentate loop. The permeate contains the protein or molecule of interest (Fig. 4-5). Separating a protein from a cell culture is similar to cell harvesting except the product of interest is the protein in the permeate. An effective cell clarification process enables the passage of the greatest amount of target molecules. To promote target molecule transmission, a wash step is often added to the cell clarification process to help flush the target molecules through the membrane (Fig. 4-6).

The level to which cells can be concentrated during cell clarification varies. Some typical values are as follows:

- *E. coli* cells: 5x concentration
- Yeast cells: 2x concentration
- Mammalian cells: 10x to 20x concentration

4.8 Lysate Clarification

The components in lysates have a propensity to foul membrane pores. To minimize pore fouling, and to enable the filter to operate under equilibrium conditions, initial lysate clarification trials often include a constant volume wash with little or no concentration. Because the starting volume is typically exchanged 5x during washing, the wash is large. Small initial volumes, 100 to 200 ml, are typical. When working with lysates, ultrafiltration filters are normally used. The small pores in ultrafiltration filters help minimize pore plugging by the submicron particles found in lysates.

The key process variables during clarification are permeate flow rate (flux) and TMP.

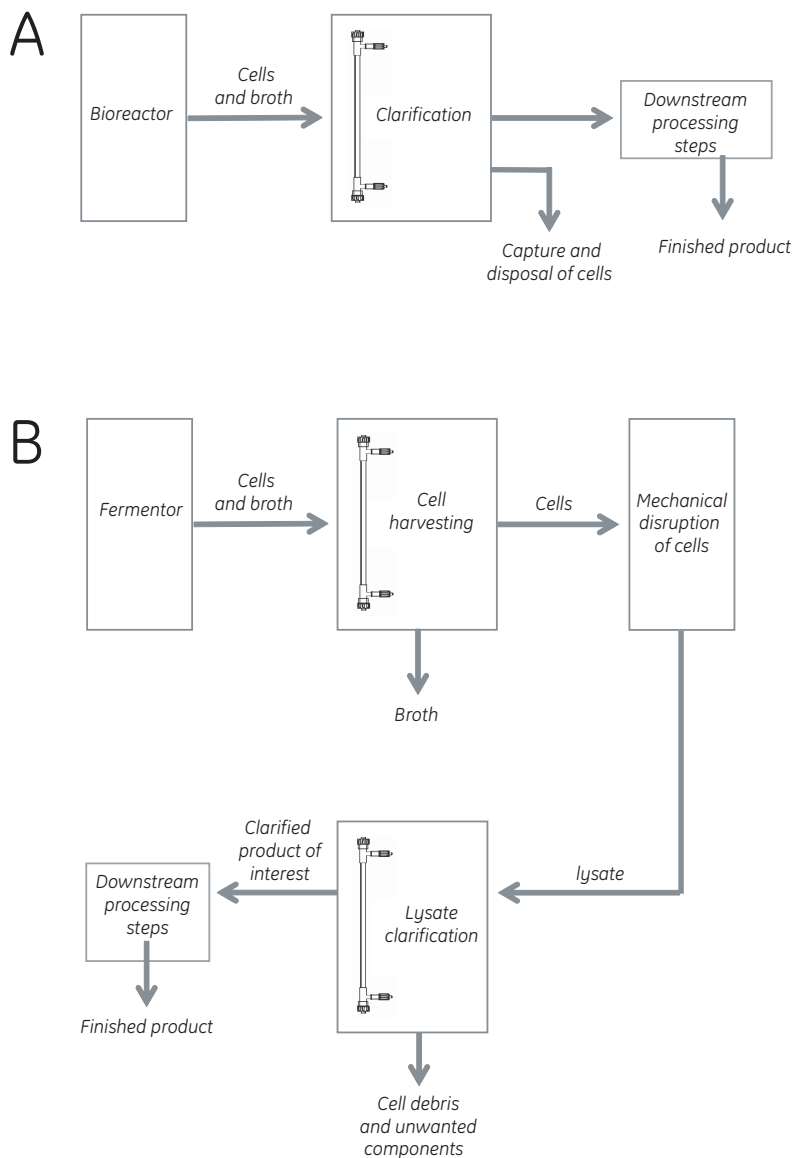


Fig 4-5. Two upstream clarification processes: (A) Separating a target protein from a cell culture, and (B) separating a target protein from a lysate.

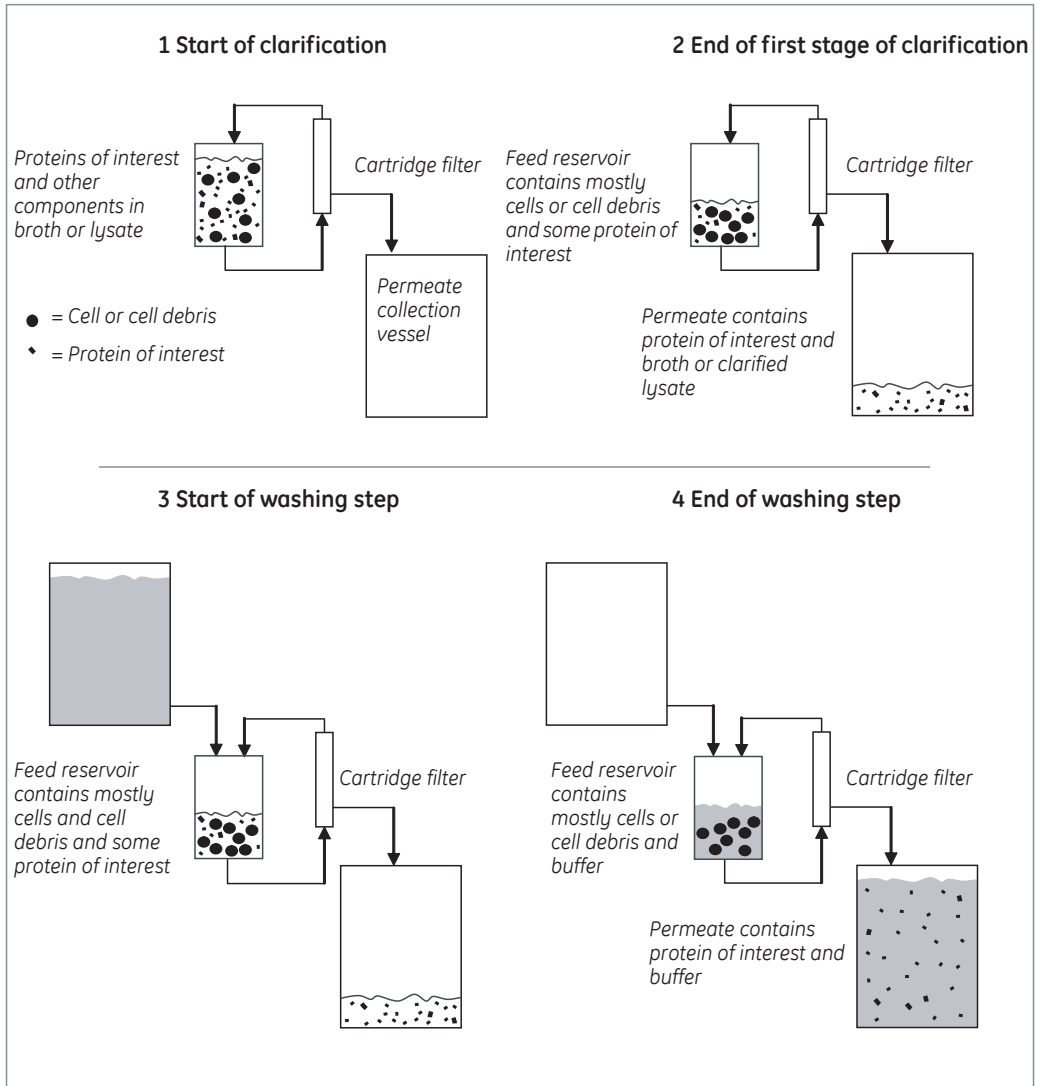


Fig 4-6. Operating principles of the clarification process.

Successful clarification of feed streams to recover target proteins requires knowledge of the starting product and the finished product specifications such as:

- Target molecule molecular weight, morphology, and robustness
- Starting volume and concentration of the target protein
- Desired finished protein concentration and volume
- Desired yield and quality (activity) of the protein
- Level of suspended solids

As with cell harvesting, rapid processing times may reduce the exposure of the target protein to shear forces, enzymatic action and temperature increases.

4.9 *Membrane and cartridge selection*

In the case of cell culture and lysate clarification, microfiltration membranes will easily retain all cells and cell debris. The key to selection is not based on retention but passage of the target molecule.

4.9.1 *Membrane selection*

When selecting membranes for clarification, smaller pore size filters resist fouling more than filters with larger pore sizes such as 0.45 or 0.65 μm . A general guideline is to select the smallest pore size ratings that is at least 10x larger than the size of the target protein in its largest state or longest dimension. When working with lysates, which can contain a wide range of particle sizes and many types of proteins and sticky cell components, choosing a small pore size can help prevent fouling of the membrane pores. Table 4-5 presents typical cartridge and membrane characteristics for common clarification applications.

4.9.2 *Cartridge selection*

The presence of particles in the feed stream requires the selection of short path length cassettes (30 to 60 cm) with large lumen diameters (0.75 to 1.0 mm). See Table 4-5 for cartridge specifications.

Type of clarification	Suspended solids and viscosity	Path length (cm)	Lumen ID (mm)	Rating (µm)	Rating (NMWC)
Monoclonal antibody from hybridoma cell culture	Low to moderate	30 or 60	1.0	0.2 0.45 0.65	- - -
Clarification of adenovirus from 293 cell culture	Low	30	0.75	0.65	-
Clarification of protein (<60 kD) from <i>E. coli</i> whole cell broth	High	30 or 60	1.0	0.1 - -	- 500,000 750,000
Clarification of 20 kD protein from <i>E. coli</i> lysate	Moderate	30 or 60	1.0	-	750,000
Clarification of 40 kD protein from <i>Pichia pastoris</i>	High	30	1.0	0.1	-

Table 4-5. Recommended cartridges for cell culture and lysate clarification.

Table 4-6 illustrates the relationship between solution variables and cartridge selection.

Solution variables	Selection considerations
Cell concentration	Determine wet cell percent to anticipate the degree of concentration that may be used. The benefit of a highly concentrated cell mass may should be balanced against the possibility of high inlet pressure requirements or the necessity of using less efficient, short (30 cm) path length cartridges.
Solids loading	For whole cells, it is not uncommon to reach 70% wet cell weight while maintaining steady state conditions. However, lysates tend to need a lower solids level to promote passage of the target material. Start with solids in the 5 to 10% range and monitor transmission as well as TMP during the concentration phase.
Size of the target material	For separations with large target material (high selectivity) it may be best to avoid any concentration but rather perform a constant volume wash from the start. Remember that using a more open membrane may require the use of short (30 cm) cartridges and permeate flow control. Use open UF membranes to clarify small proteins from either whole cell broths or lysate streams.
Shear sensitivity	If the feed stream is particularly shear sensitive and the recirculation flow rate is reduced, it may be necessary to lower the permeate flow rate (when using permeate flow control) to optimize throughput.
Other variables	Selection considerations
Time constraints	Increased membrane area and larger housing size shorten production time.
Heat sterilization	Choose autoclavable or steam-in-place models for scale-up.

Table 4-6. The influence of process variables in selecting a cross flow cartridge.

4.10 Filter and system preparation

The steps for filter and system cleaning are as outlined in the previous section on cell harvesting.

4.11 Operating parameters

4.11.1 Permeate flow control

In clarification processes, as in cell harvesting, the flux is often high even at low TMP values and steps should be taken to decrease the flux to prevent premature fouling of the membrane. Please refer to the section on cell harvesting for more details. Table 4-7 describes typical starting conditions for the clarification of different cell types.

Bacterial fermentation-clarification of target protein expressed extracellularly	Mammalian cell culture -Mab clarification	Yeast fermentation-clarification of target protein expressed extracellularly	Adeno-associated virus-clarification from 293 or HeLa cells	Clarification of VLP from a yeast lysate
5X concentration followed by a 3 to 5X diafiltration.	10X concentration followed by 3X diafiltration.	Partial concentration 1.5 to 2X at best followed by 3 to 5X diafiltration.	5X concentration followed by 5X wash.	First, try a constant volume diafiltration process with no initial concentration with high shear and permeate flow control set at 20 LMH. With good transmission, a 5X wash may suffice.
For large target proteins, use microfiltration membranes with permeate flow control set at 20 to 30 LMH. For smaller molecules, use 750 or 500 kD UF membranes with unrestricted permeate flow and TMP readings at 1 to 1.5 bar.	Use 0.2 or 0.45 microfiltration membranes with permeate flow control set at 30 LMH. No retentate back pressure. This process description is for the removal of cells and optimal recovery of the target protein.	Membranes rated at 750 kD UF and 0.1 micron MF have worked well with unrestricted permeate flow. If the cell density is quite high, closely monitor the inlet pressure to avoid over concentration.	Operate at low shear conditions using 0.65 micron membranes and permeate flow control set at 20 to 30 LMH.	

Table 4-7. Recommended starting point for developing process conditions for clarification.

4.12 Three examples of clarification strategies

The following examples illustrate the development of process conditions with mammalian cells, bacterial cells and yeast.

4.12.1 Mammalian cells

Recently developed therapeutic proteins are derived from cell culture sources. These are most often grown with mammalian cell lines in highly purified media. Although there are a variety of cells that are suitable and cell densities range from 10^6 to 10^7 cells per ml, the clarification process is similar for each type.

Membrane and cartridge selection

If the protein of interest is an antibody, 0.2, 0.45, or 0.65 micron membranes can be chosen. Permeate turbidity will be slightly lower if 0.2 micron filters are used. The 0.65-micron rating usually provides the best throughput. These cartridges can be tested with feed solution to determine which rating provides the best overall performance. The above microfiltration membranes are available in only one fiber diameter, and the only remaining variable is the cartridge path length, 30 or 60 cm. Initial testing should begin with the 30-cm path length to help maintain low TMP readings. When the appropriate membrane and operating conditions have been chosen, additional testing using the 60-cm path length may be used to determine if this design is suitable for scale up.

Process conditions and monitoring

The circulation flow rate should be set up to 4000 sec^{-1} . If tests show damage to the cells, the circulation flow rate should be reduced. The permeate flow rate should be set to 30 to 50 LMH. Using these conditions, the initial TMP will begin at approximately 70 mbar. Since the cells are completely retained, and the protein will initially pass through the membrane quantitatively, the objective of testing should be to determine the filtration capacity. As a general rule, once the TMP has increased by a factor of 4 to 5 from the initial reading, the membrane is exhausted. In this example, working with permeate flow control set at 30 LMH, if the TMP begins at 70 mbar, when the TMP reaches 250 to 350 mbar the cartridge capacity has been reached.

Using the change in TMP as an indicator, it is possible to compare a variety of membrane ratings and process controls. Once a set of standard conditions has been adopted, the filtration efficiency can also be studied as a function of the cell culture process. For example, with low cell viability, membrane throughputs working with a $0.45 \mu\text{m}$ membrane might be as low as 50 L/m^2 . With healthy cells operating under the same conditions, the throughput might be as high as 120 L/m^2 . Operating at a higher flux rate usually decreases throughput capacity.

For high yields working with monoclonal antibodies derived from CHO cells, it is normally possible to concentrate the cells 10X and follow with a 3X to 5X wash.

Using the 30-cm long Start AXM hollow fiber cartridge with 50-cm², and with a starting volume of 400 to 500 ml, the process time will be approximately two hours.

4.12.2 Bacterial cells

E. coli and related bacterial cells have been used for many years for the expression of a wide range of recombinant proteins, vaccines, and enzymes. The fermentation times can range from under a day to a week. Due to the short doubling time of these cells, prolonged fermentation can result in a significant cell mass. Moreover, the nutrient media is usually much more complex and less purified than the material used with mammalian cells. As a result, separating these cells from the target protein can be a more complex process.

Membrane and cartridge selection

For relatively small proteins, < 40 kD, the open ultrafiltration membranes rated at 500 kD and 750 kD should be tested first. These membranes will provide a stable flux rate and resist rapid fouling. For large proteins, microfiltration membranes rated at 0.1 or 0.2 μm should be used, but only in conjunction with permeate flow control. In order to make a comparison of any of these selections without a significant contribution from a secondary rejection layer, uniform operating conditions with a relatively high shear rate in the circulation flow and low TMP should be used. Initial testing should use the 1 mm inside diameter fiber design with a 30 cm path length. When the membrane and operating conditions have been chosen, additional testing using the 60-cm path length may be used to determine if this design is suitable for scale up. Several full scale production processes have successfully utilized the 110 cm ultrafiltration cartridge design with *E. coli* fermentation processes.

Process conditions and monitoring

Unlike fragile mammalian cells, bacterial cells can withstand significant shear forces without damage. Test results have shown that high circulation flows with 12,000 to 16,000 sec^{-1} shear rates provide better transmission of the target protein and more stable flux rates. Insufficient shear force or excessive TMP will cause the formation of a secondary rejection layer on the membrane surface that will prevent the passage of the target protein. Therefore, initial testing will require a reliable assay to establish a stable process with good yields. With feed streams containing a high cell mass and a large target molecule, testing should begin with the permeate flow control set as low as 10 to 20 LMH. When working with ultrafiltration membranes, the TMP should be gradually increased to see if there is a proportionate and stable increase in flux. With microfiltration membranes, the flux rate should be gradually increased while monitoring the TMP. If the TMP increases over time, the flux rate should be adjusted to a lower setting until it remains stable. Even with a relatively high starting cell mass, it is often possible to perform a 5X concentration without sustaining a significant increase in the pressure drop along the cartridge. If the inlet pressure begins to

rise abruptly, it is normally not due to cartridge plugging but to the increased viscosity of the feed, and additional concentration is not advised. With initial testing, a 2X increase in the pressure drop should be used as an upper limit. The constant volume wash should be initiated without interrupting the circulation flow. If the flux rate has decreased by more than 4X, it is advised to temporarily open the back pressure valve and shut off the permeate flow. This technique may help to diminish the effects of the secondary rejection layer.

Due to the relative difficulty of working with bacterial cell suspensions, testing should emphasize selecting the membrane that provides the best long term transmission of the target protein coupled to a process sequence that maintains the process in an equilibrium status.

With regard to capacity, testing should start at 25 L/m². Using a 5X concentration followed by a 5X wash with a 50-cm² test cartridge and 150ml of starting material, the process time is expected to be 2 to 3 hours.

4.12.3 Yeast

Pichia pastoris and other types of yeast have been extremely popular for expressing target proteins. Often a successful fermentation will result in a highly viscous material with as much as 50 percent cell mass. This represents a challenge for any of the candidate clarification technologies. However the target proteins are usually small. Moreover, the hollow fiber technology is linearly scalable and does not require pre-dilution in order to provide good yields.

Membrane and cartridge selection

The most popular membranes to ensure good passage of target proteins as large as 70 kD molecular weight have been the 0.1 micron microfiltration and 750 kD ultrafiltration membranes. Larger proteins have been successfully processed at full scale with the 0.2 micron microfiltration membranes. With such a high initial viscosity, there is even greater need to use the most dense membrane that will effectively pass the target protein. More open membranes working with high inlet pressures will result in cells being trapped on the membrane surface near the cartridge inlet. Inlet pressures will rise and the process will fail. The 1 mm fibers with the 30 cm path length are used exclusively with viscous yeast feed streams. Moreover, the circulation flow rate rarely exceeds 4,000 to 6,000 sec⁻¹ in order to keep the inlet pressure readings less than 0.7 bar. Due to the high viscosity of the feed stream, it is critical that all characterization testing be done while operating in total recycle. Even a slight increase in the solids concentration will result in a significant increase in the pressure drop.

Process conditions and monitoring

When working with the 750 kD ultrafiltration membrane, the circulation flow rate should be kept constant while gradually increasing the TMP to see if there is a proportionate and stable increase in flux. If the increased TMP only provides a

partial increase in flux, the TMP should not be increased as this may result in a drop in protein transmission, which will necessitate extensive washing. With either the 0.1 or 0.2 μm microfiltration membranes, the flux rate should be gradually increased while monitoring the TMP. As in the earlier example, if the TMP increases over time, the flux rate should be adjusted to a lower setting until it remains stable. The TMP readings with these microfiltration membranes will probably remain below 0.34 bar throughout the process. Unless the starting cell density is quite low, < 35%, it is unlikely that the process will allow any initial concentration of the feed stock. Instead, it is best to operate under a constant volume wash mode from the beginning. Since as much as 50% of the feed is actually cells, the wash volumes double their effectiveness. High yields are possible using only 2.5X wash volumes. Moreover, because the membrane is operating under equilibrium conditions, flow rates and pressure readings should remain constant.

Initial testing should be directed at selecting the membrane with the best passage of the target protein. This is usually the membrane that also provides the highest flux rate. Optimization of the operating conditions will involve minor adjustments to the pressure readings and/or flux rates. Capacity will be a function of the protein transmission as this will lead to the determination of the required wash volumes. Effective processes will be between 2.5 and 5X wash volumes. Flux rates can range from 15 to 60 LMH. When working with high cell density feed streams, it is possible to achieve throughputs of 80 L/m² based on the starting material. Using a 50 cm² hollow fiber cartridges and a target 50 LMH flux rate, 400 ml of feed material with a 2.5X wash objective will require a process time of four hours for a complete test.



5 Concentration and Diafiltration

5.1 Introduction

CFF is used extensively in conjunction with chromatography to concentrate and purify proteins for use in pharmaceuticals. The multi-step process of CFF concentration/diafiltration and chromatographic purification of proteins is referred to as downstream processing. Most downstream concentration and diafiltration processes use membrane cassettes. The main steps in the downstream purification process are illustrated in Fig. 5-1 and Fig. 5-2.

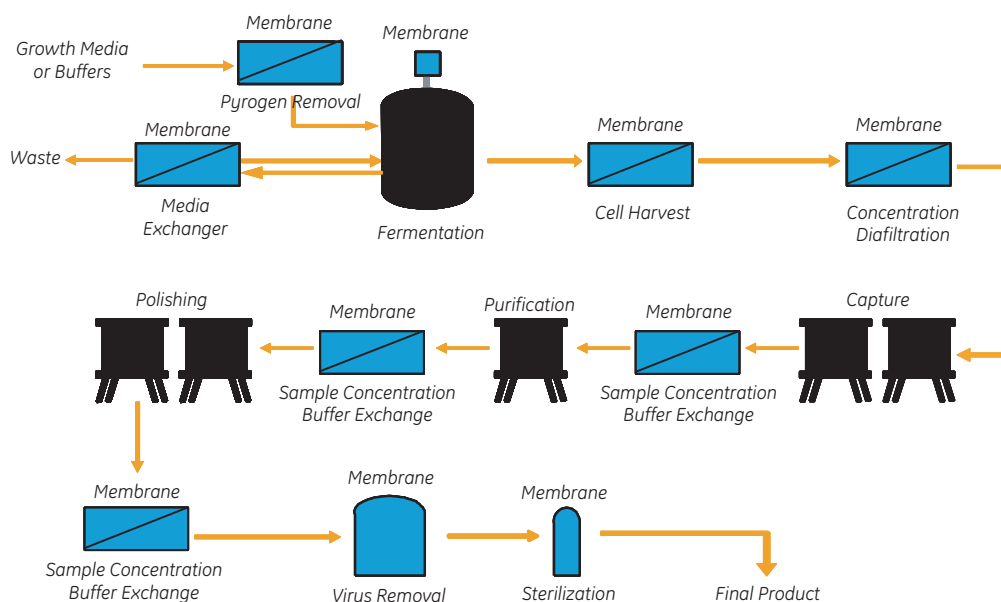


Fig 5-1. Downstream steps in the purification of IgG.

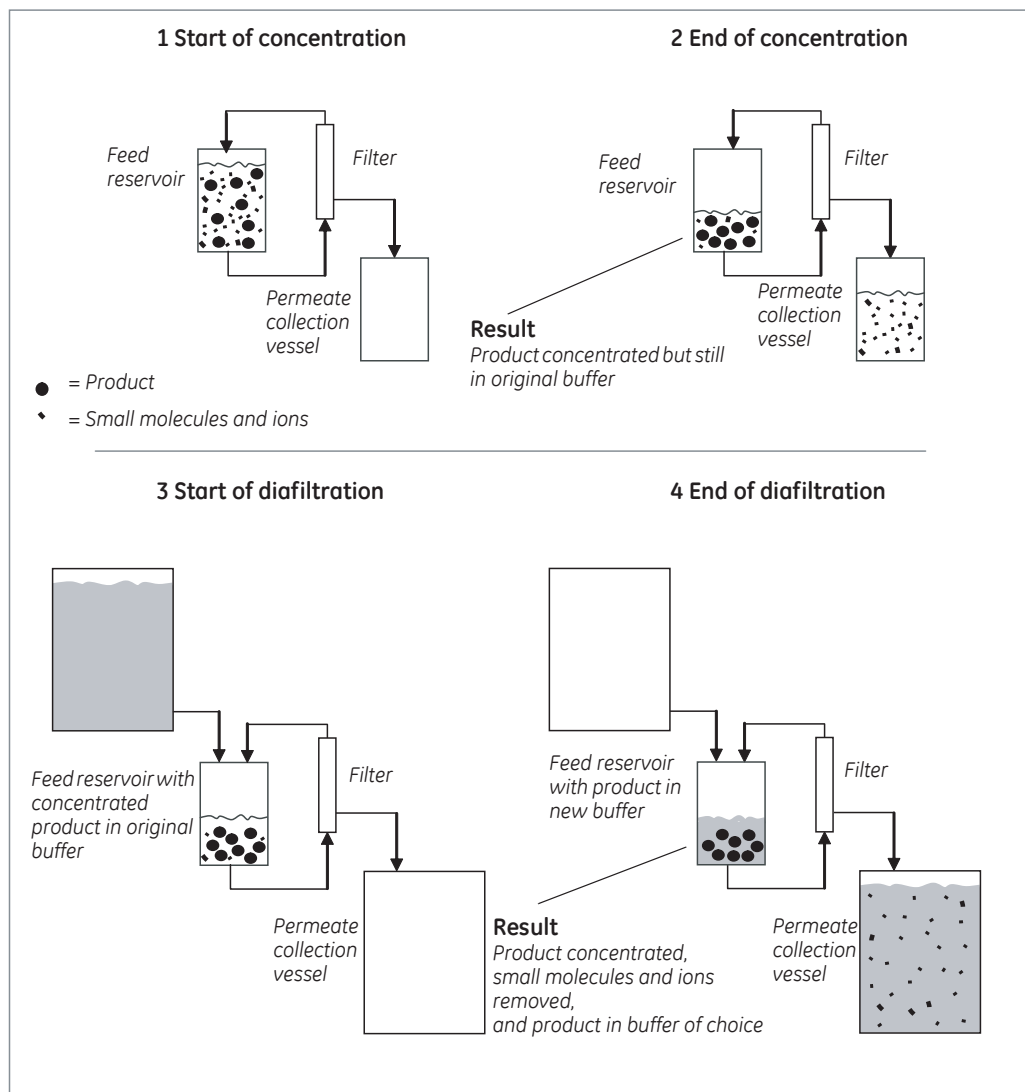


Fig 5-2. Comparison of concentration and diafiltration processes.

5.2 Product and process considerations

Successful protein concentration and diafiltration using ÄKTAcrossflow system relies on specifying the pre- and post-concentration product as follows:

-
- The characteristics of the target protein (size and shape, solubility, shear sensitivity, temperature sensitivity, ionic condition, and the role of the chromatography step)
 - The starting characteristics of the protein solution (pH, ionic condition, solubility, compatibility with the chromatographic step) and the target characteristics for the next step
 - The starting volume and concentration of the target protein
 - The desired finished concentration and volume
 - Process time and cost
 - Diafiltration volumes
 - The desired yield and quality (stability) of the target protein

5.3 Diafiltration

The goal of diafiltration is buffer exchange to optimize the next chromatographic purification step or for final formulation of the protein solution.

From an operational perspective the goal is to minimize consumption of the diafiltration buffer and to shorten diafiltration processing time. The diafiltration process usually consists of concentration followed by continuous diafiltration.

5.3.1 Efficiency

To increase process efficiency, the product concentration can be increased before diafiltration, thereby reducing the amount of buffer required to achieve a specific diafiltration factor. Alternatively flux can be increased during diafiltration to shorten processing time. In both cases, crossing the optimum process point results in decreasing returns. For example, if the protein solution becomes too concentrated prior to diafiltration, the flux decreases, offsetting the benefits of reduced buffer consumption.

5.3.2 Discontinuous diafiltration

Discontinuous diafiltration is a process where the protein solution is repeatedly concentrated and re-diluted. It is less efficient than continuous diafiltration because of the extra volume of buffer required.

5.3.3 Sequential diafiltration

Occasionally it may not be possible to exchange the existing buffer with a new buffer directly without damaging effects to the target protein. In sequential diafiltration, several buffer formulations, moving from weaker to stronger chemical solutions are introduced sequentially to the product to achieve the final buffer exchange.

5.4 Membrane and cassette selection

5.4.1 Membrane selection

A membrane with too large an NMWC will allow the molecule of interest to pass through and significantly reduce yields. Conversely a membrane with too small an NMWC will reduce flux and lead to slower processing times, oversized systems, increased capital cost, and plant space requirements, working volume and hold-up volume. For typical membrane- based cross flow applications, the membrane pore size selection is based on the size of the target molecule.

The general guideline for selecting a membrane for product concentration is to start with a NMWC that is 3 to 5 times smaller than the target molecule. For example, a 50 kD or 30 kD membrane would be a suitable choice to retain IgG (160 kD), and a 30 kD or 10 kD membrane would be a suitable choice for albumin (66 kD).

5.4.2 Cassette selection

Kvick Start cassettes are used for downstream processes such as concentration of proteins and diafiltration before chromatography and for final concentration and purification of post chromatography product. Kvick Start cassettes incorporate polyethersulfone membrane and when operated correctly, a high level of protein recovery can be expected. As with any polymeric membrane, a low level of non specific protein binding is possible.

5.5 Device and system preparation and cleaning

Safety and operating instructions are included with all cassettes and these should be followed when preparing the cassette for use. A new cassette contains an aqueous solution of 0.1 to 0.2 N NaOH and 20% to 22% glycerin. Before using a new or previously stored Kvick Start cassette the storage solution must be

removed. In addition, some applications require the completion of other preparatory steps before using the cassette.

The main preparatory steps are summarized below.

- Rinsing the Kwick Start cassette.
- Rinsing storage solution from a previously used Kwick Start cassette.
- Measuring the water flux before and after use. Determines the cleaning effectiveness and monitors the Kwick Start cassette's performance from run to run.
- Sanitizing the Kwick Start Cassette. Kwick Start cassettes and ÄKTAcrossflow system can be treated with sanitizing agents such as 0.5N NaOH.
- Conditioning the system with buffer. This exposes the system's wetted parts to an appropriate buffer before introducing the product. The buffer can also bring the system to the proper operating temperature before processing begins.

All these steps are included in the method wizard of ÄKTAcrossflow system software.

5.6 *Operating conditions for Kwick Start cassette*

The following operating conditions are recommended starting points for downstream concentration trials:

- Typical operating flow rate: 25 to 50 ml/min/cassette
- Typical transmembrane pressure: 1.7 to 2.4 bar (25 to 35 psi)
- Maximum operating pressure: 4 bar (60 psi) at 23°C
- Maximum operating temperature: 50°C at 2 bar (30 psi)
- Recommended pH operating range: 1 to 14
- Kwick Start non-recoverable (drained) volume: less than 3 ml

5.7 *Concentration factor*

The maximum available concentration factor is limited by the ratio between starting volume and minimum working volume. In addition, over concentration of protein can lead to inefficient diafiltration due to membrane polarization effects and to protein precipitation.

5.8 Optimization of TMP

TMP excursions are an important part of process optimization. Increasing TMP when ultrafiltering pure water results in a proportional increase in flux. With a process fluid that contains solutes there is a similar pressure dependent region of operation where an increase in TMP corresponds to an increase in flux, but there is also a pressure independent region, where an increase in pressure does not lead to an increase in flux (Fig. 5-3). The pressure independent region is a result of the build up of solutes at the surface of the membrane, called a gel layer, which creates resistance to flow. This gel layer can be reduced by increasing the crossflow.

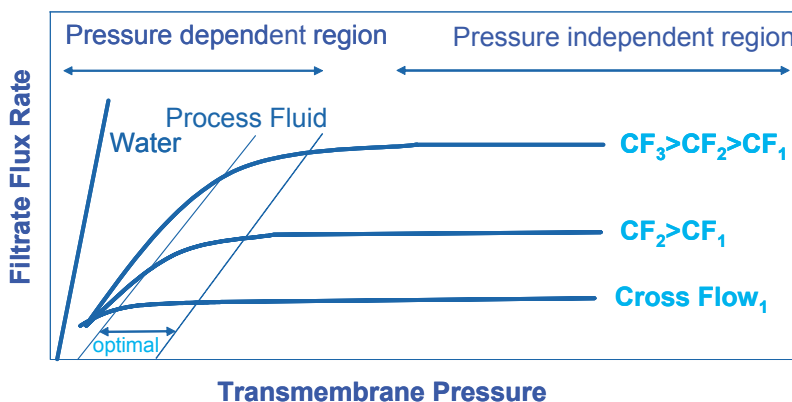


Fig 5-3. TMP excursions are an important part of process optimization.

5.8.1 Concentration

The standard procedure is to perform a TMP excursion experiment in which a series of TMP setpoints is measured at different crossflow rates. From these experiments the effect on flux is evaluated, and optimal crossflow and TMP may be identified. ÄKTAcrossflow software contains a method Wizard, where the user can input the desired parameters and the software creates the method to perform the experiment. The standard method may be used without any changes, or may be easily modified to meet most processing needs.

Fig. 5-4 is an example of one of the result files of a TMP excursion displayed in the UNICORN Evaluation window for Result files.

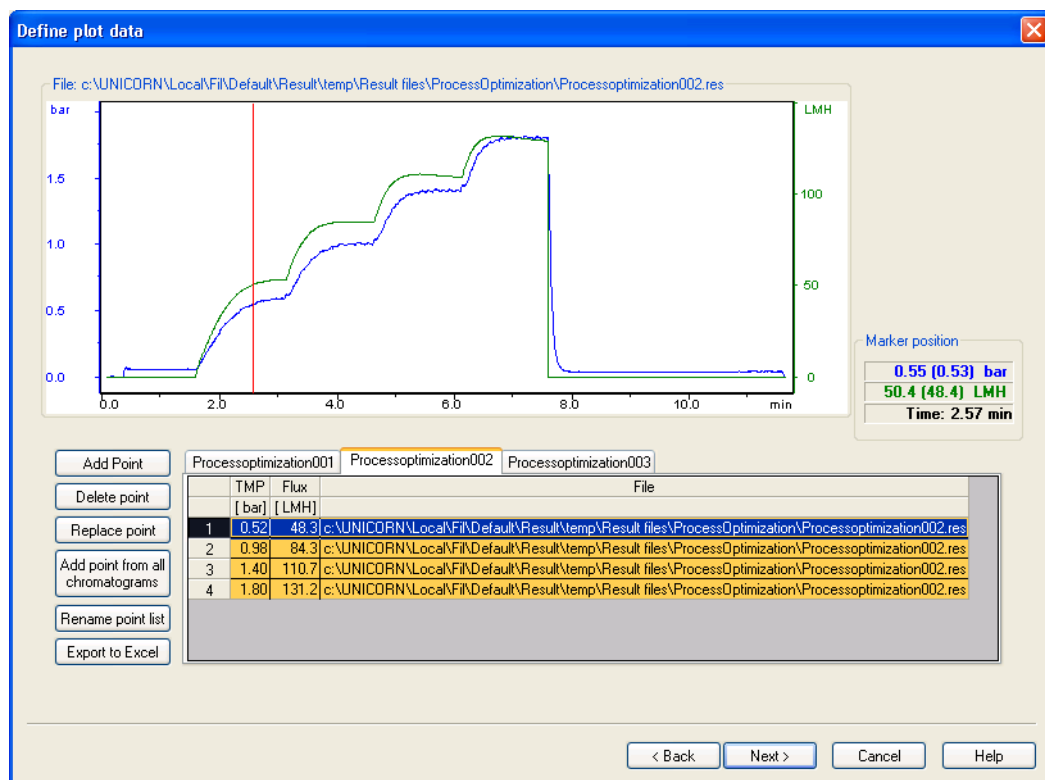


Fig 5-4. Result file of a typical TMP excursion.

Fig. 5-5 shows the results of CF-TMP optimization for a solution of BSA at a concentration of 30g/L (original concentration) and 150g/L (target concentration). In this example 6 TMP points are measured at 3 CF rates in total recycle mode. The data were analyzed by the Evaluation module in ÅKTAcrossflow software and plotted as TMP excursion curves. In this example, it can be seen that the excursions done at low protein concentration indicate that all TMP setpoints operate in the pressure dependent region. At high protein concentration, the pressure independent region of operation is beginning to be seen and increasing the crossflow results in a significant increase in flux.

This enables a crossflow rate to be chosen which gives a high flux value for a reasonable process time.

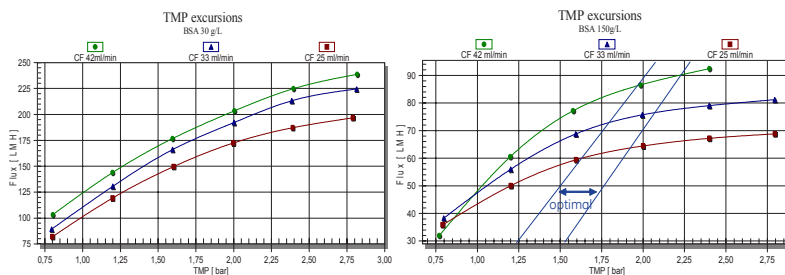


Fig 5-5. TMP excursion results at two concentrations of BSA.

5.8.2 Diafiltration time optimization

In a concentration process, the optimized crossflow and TMP conditions established above can be used to identify the diafiltration point (the point which provides the fastest buffer exchange), and optimal buffer consumption. A typical result file for diafiltration time optimization is shown in Fig. 5-6.

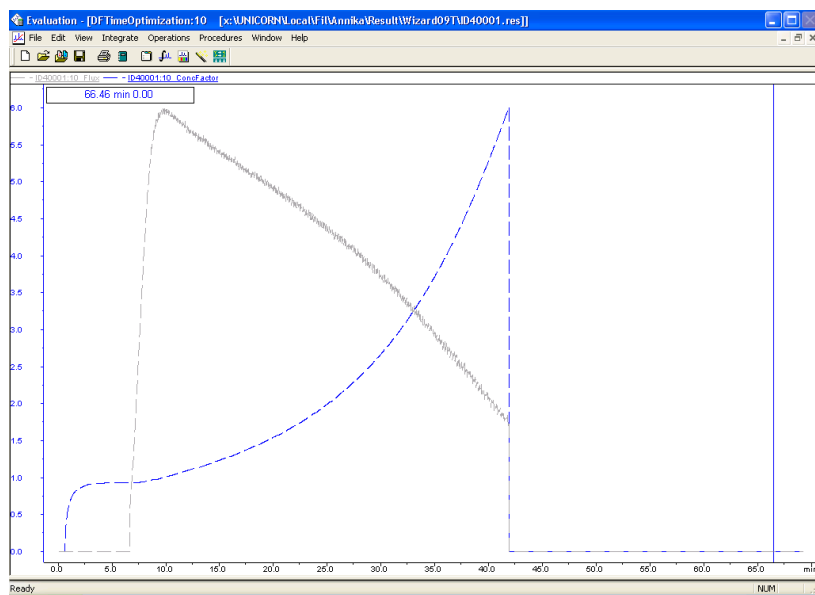


Fig 5-6. Result file for diafiltration time optimization. Flux is the grey curve, concentration factor is the blue curve.

A curve of Flux*concentration factor versus concentration factor can be created in the Evaluation module which enables the optimization of diafiltration time (Fig. 5-7). The highest value on the y axis at the highest concentration identifies the fastest diafiltration with the lowest buffer consumption. This example also shows that diafiltration takes the same time if performed at four times or five times concentration, because the decrease in retentate volume at five times concentration is offset by the decrease in flux.

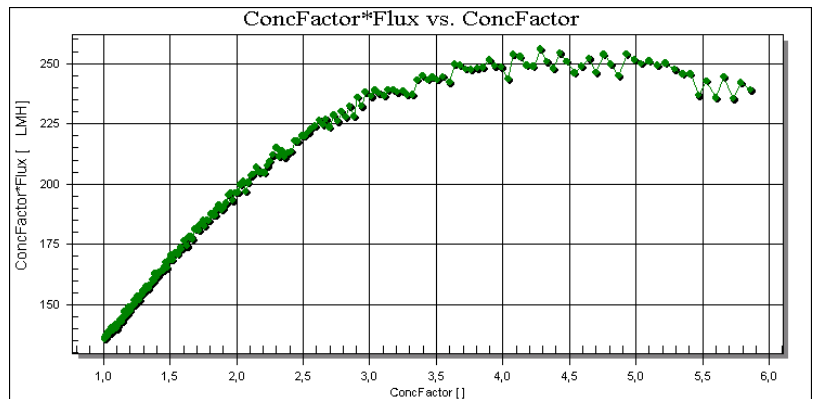


Fig 5-7. Diafiltration time optimization.

5.8.3 Diafiltration factor

The Diafiltration factor (DF) is the percentage of original buffer remaining in the feed:

$$\text{Diafiltration factor} = \text{Sample Volume} / \text{Buffer Volume}$$

The volume of buffer required to achieve a desired diafiltration factor can be calculated using the following formulae:

$$\text{Continuous diafiltration: } C_f/C_o = (1-\beta)^{DF/\beta}$$

$$\text{One shot diafiltration: } C_f/C_o = 1/(DF+1)$$

$$\text{Discontinuous diafiltration: } C_f/C_o = 1/2^{DF}$$

Where:

- C_f = final concentration
- C_o = Original concentration
- β = sample turn over ratio per unit time

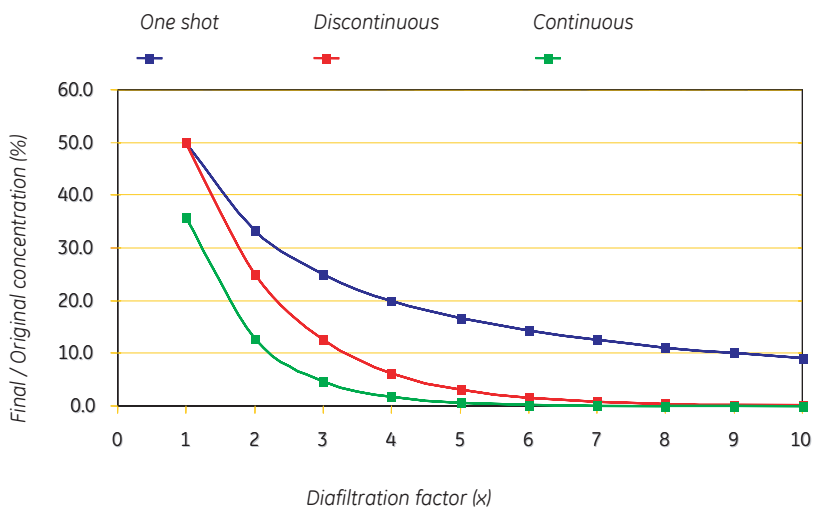


Fig 5-8. Effect of diafiltration methods on the buffer concentration.

6 Applications

6.1 *Purification of β -glucosidase from a *Pichia pastoris* cell culture broth using microfiltration*

6.1.1 Objective

The objective of this application was the clarification of a yeast cell suspension and the recovery of β -glucosidase in the permeate. *P. pastoris* expresses β -glucosidase extracellularly so the protein is in the culture broth. β -glucosidase is a complex consisting of five subunits with an approximate molecular weight of 400 kD. *P. pastoris* cells can be grown to a very high cell density, in this application the dry cell weight content was ~17% (equivalent to ~45% wet cell weight). This high solids content can create problems at higher crossflow rates.

When using hollow fibers, the cross flow rate is converted into a shear rate which represents the crossflow per fiber and fiber diameter. Since the fibers are relatively open they rely on a high crossflow rate to create enough turbulent flow to prevent the cells from accumulating at the membrane surface. The crossflow rate or shear rate and the amount of restriction on the permeate flow are crucial parameters to processing such high density cell suspensions without immediate membrane fouling and clogging of the filter. To find the optimal parameters CFF experiments were carried out on an ÄKTAcrossflow system using 50 cm² Start AXM hollow fiber membrane cartridges.

6.1.2 Process Optimization

The strategy for process optimization was as follows:

- Membrane selection
- Optimization of shear-flux settings taking into consideration shear limiting factors such as protein sensitivity to shear damage and feed pressure
- Find the number of retentate washes needed for improved protein recovery

In microfiltration applications there is no equivalent to TMP excursions, so full process runs have to be completed under different experimental conditions, and evaluated by analyzing the protein recovery. However with the Method Wizard, a method can be generated in minutes.

6.1.3 Membrane selection

The filter pore size chosen should be the one that retains the solids while yielding the highest recovery of the target protein in the shortest process time. In the following example, four membrane pore sizes were screened: 750 kDa, 0.1 μm , 0.2 μm and 0.45 μm . Different shear rates were also tested for each membrane pore size to find the combination that gave the highest recovery. The 750 kDa membrane gave a very low recovery in initial experiments and was not included in further tests.

The pore sizes were tested with two different methods:

- No concentration and 4 times washing of the retentate
- 1.4x concentration and 4 times washing of the retentate

The washing steps are essential for improved protein recovery, particularly when clarifying a high solid content sample with little or no possibility of an initial concentration step.

Since, as can be seen in Table 6-1, approximately 100% protein recovery was obtained using the 0.45 μm filter this membrane was selected for further process optimization.

Membrane pore size	Shear rate (1/s)	Recovery (%)	
		No concentration	1.4x concentration
0.1 μm	5500	58	60
0.1 μm	8000	56	-
0.2 μm	4000	54	71
0.2 μm	4500	67	72
0.45 μm	4000	62	~100
0.45 μm	6000	70	-

Table 6-1. Membrane selection.

6.1.4 Optimization of shear-flux settings

For the membrane pore size of 0.45 μm , the effect of shear rate on maximal achievable flux was studied using methods with no concentration and 1.4 x concentration. A higher shear rate results in increased flux which shortens process times. However, at a shear rate of 6000 (1/s) the concentration of 1.4 x could not be achieved because the feed pressure exceeded its operating limit. With no concentration at this shear rate, a flux rate of 55 LMH could be achieved. Increased flux gives shorter process time but that has to be weighed against the risk of shear damage to a protein of such high molecular weight (400 kD) and that no concentration was possible. A concentration would decrease the washing time and buffer consumption. Therefore results showed that the optimal parameters were to concentrate 1.4 x and run at a shear rate of 4000 (1/s) and a flux of 35 LMH.

Shear rate (1/s)	Flux (LMH)	
	No concentration	1.4x concentration
4000	45	35
6000	55	-

Table 6-2. Shear flux optimization results.

6.1.5 Optimization of retentate wash for protein recovery

The final step in the process optimization was to evaluate the effect of washing on product recovery (Fig. 6-1). As already described due to the high cell density of the sample it was not possible to achieve a high concentration of the starting material, and the process would therefore largely consist of washing the retentate with buffer to promote protein passage.

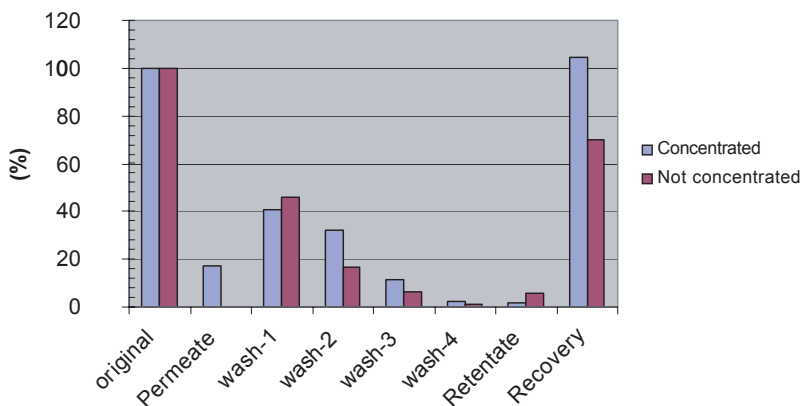


Fig 6-1. Evaluation of ion effect of washing on product recovery.

Using the process parameters established earlier, the protein recovery was measured after each of four washing steps for both concentrated and unconcentrated samples. As seen in Fig. 6-1 the fourth wash did not contribute much to the overall recovery of the target molecule so three washes is enough for this process.

6.1.6 Conclusion

For recovering β -glucosidase from this high cell density *P. pastoris* culture using microfiltration, the following optimal process parameters were identified:

- Use a membrane pore size of 0.45 μm
- Re circulate feed at a shear rate of 4000 (1/s)
- Perform the process with a flux setpoint of 35 LMH
- Concentrate to a factor of 1.4 x (to 21% dry cell weight)
- Wash the cells with three retentate volumes of buffer

Fig. 6-2 shows the results from a complete run using the parameters above.

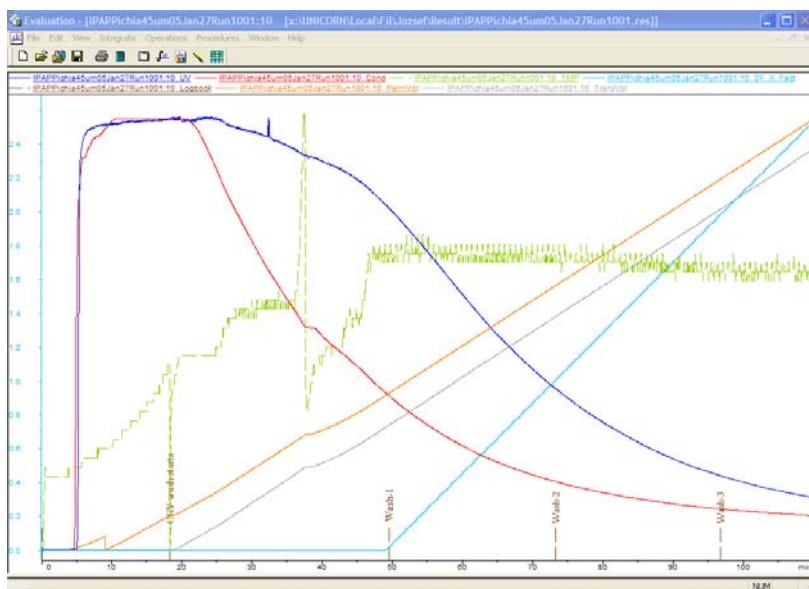


Fig 6-2. Clarification of *P. pastoris* culture using 0.45 μm Start AXM cartridge. This graph view gives the possibility to monitor for example the decrease in UV, i.e. absorbance at 280 nm, as the target protein is transferred to the permeate.

6.2 Purification of Green Fluorescent Protein-His (GFP-His) from an *Escherichia coli* cell homogenate

6.2.1 Objective

The objective of this application was clarification of the *E. coli* strain BL21 (DE3) homogenate (removal of cells and cell debris) and the recovery of the target protein in the permeate. GFP-His is an intracellular protein which is over expressed in the cells and occurs as monomers with a molecular weight of approximately 30 kD.

It has been observed that GFP-His adsorbs to cell debris at pH values lower than 8.2 so the pH of the homogenate prepared from 25 g/l cell suspension was adjusted to 8.3. Hollow fiber cartridges are often chosen for *E. coli* homogenate clarification because of their open feed channel design. Optimization of the clarification process involves selecting a filter pore size and operating conditions that retain solids while yielding the highest recovery of the target protein in a specified process time. CFF experiments were carried out on ÄKTAcrossflow system using 50 cm² Start AXM hollow fiber cartridges.

6.2.2 Process Optimization

The strategy for process optimization was as follows:

- Membrane selection
- Optimization of shear-flux settings
- Find the number of retentate washes needed for improved protein recovery

6.2.3 Membrane selection

The objective was to choose the smallest membrane pore size which provides an excellent recovery of target protein. Smaller pore sizes reduce the tendency for particles to become embedded in the membrane. Three membrane pore sizes were screened: 750 kD, 0.1 µm and 0.2 µm, at a cell concentration of 25 g/l. The sample was concentrated three times and then washed three times with buffer. As mentioned previously, in microfiltration applications full process runs have to be completed under different experimental conditions, but the Method Wizard in ÄKTAcrossflow software quickly creates the desired process methods.

Table 6-3 summarizes the results of the membrane screening experiments. Protein recovery was 75% and 83% respectively for membranes with pore sizes 0.1 µm and 0.2 µm, and these were selected for further investigations

Pore size	Concentration factor	Wash volume	Protein recovery
750 kD	3	3X	61%
0.1 μm	3	3X	75%
0.2 μm	3	3X	83%

Table 6-3. Results of pore size screening with GFP-His.

6.2.4 Optimization of shear-flux settings

The objective of these experiments was to determine the maximum flux rate for a given shear rate, accessed by TMP stability. A constant shear rate was selected (in the example shown in Fig. 6-3 the shear rate was 8000 sec^{-1}), and the flux increased in a stepwise manner, while monitoring the TMP. These experiments were done at the target concentration of the sample and the permeate was recycled back into the retentate. A rapid increase in TMP indicates reduced membrane permeability due to gel layer formation. The process was repeated for shear rates of 8000 and 16000 sec^{-1} , for both the 0.1 and $0.2 \mu\text{m}$ membrane pore sizes and the data analyzed. Fig. 6-3 shows the results of an experiment using a shear rate of 8000 sec^{-1} and a pore size of $0.1 \mu\text{m}$. In this test it can be seen that the maximum flux set point which results in a stable TMP is 40 LMH .

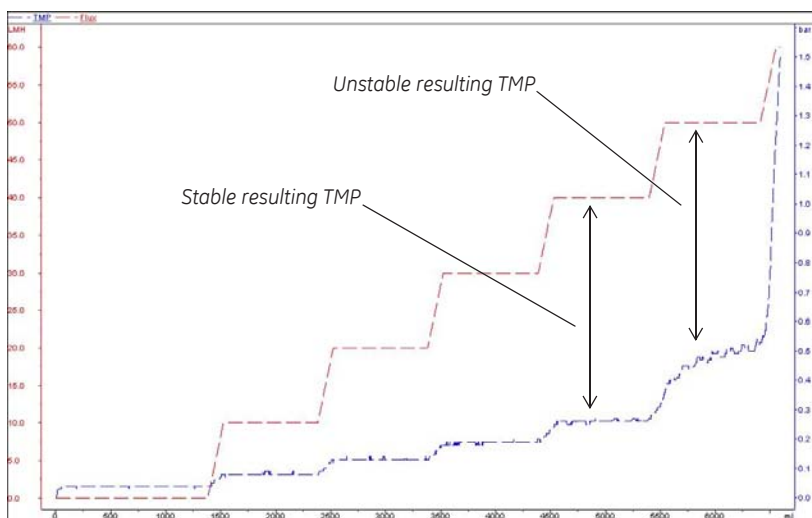


Fig 6-3. Results of a flux excursion to determine the maximum flux set point for a given shear rate.

When using the 0.1 µm membrane pore size with a shear rate of 16000 sec⁻¹, a high flux (45 LMH) could be achieved, and the recovery was relatively high (83%), see Table 6-4. The highest product recovery (93%) was reached using the 0.2 µm pore size at this high shear rate, but the flux was low (20 LMH). The high protein recovery is offset by the low flux and resulting longer process time. This however could be compensated by a larger membrane area, but would result in higher costs in pump capacity and membranes. In summary, for the fastest process time and acceptable recovery the 0.1 µm membrane pore size was chosen at a shear of 16000 sec⁻¹.

Membrane pore size ratings	Shear rate (1 sec ⁻¹)	Flux (lmh)	Protein transmission (% of total content)
0.1 µm	10 000	35	70
0.1 µm	14 000	40	85
0.1 µm	16 000	45	83

Table 6-4. Results of flux optimization.

6.2.5 Optimization of retentate wash for protein recovery

The effect of washing buffer on protein recovery is shown in Table 6-5. During the three times concentration 50% - 60% of the total GFP-His content passed into the permeate depending on the membrane pore size used. Increasing the number of washes contributes to higher recovery, but the amount recovered after 3 washes is small. The amount of additional protein recovered should be balanced against the time needed for the extra wash steps.

Membrane pore size ratings	Permeate	Wash 1	Wash 2	Wash 3	Wash 4
0.1 µm	53%	14%	8%	5%	3%
0.2 µm	60%	16%	9%	5%	3%

Table 6-5. Results from GFP-His wash volume - recovery study.

A full run was completed with the optimal process parameters and the result is shown in Fig. 6-4. The run was repeated three times in order to confirm the robustness of the selected process parameters.

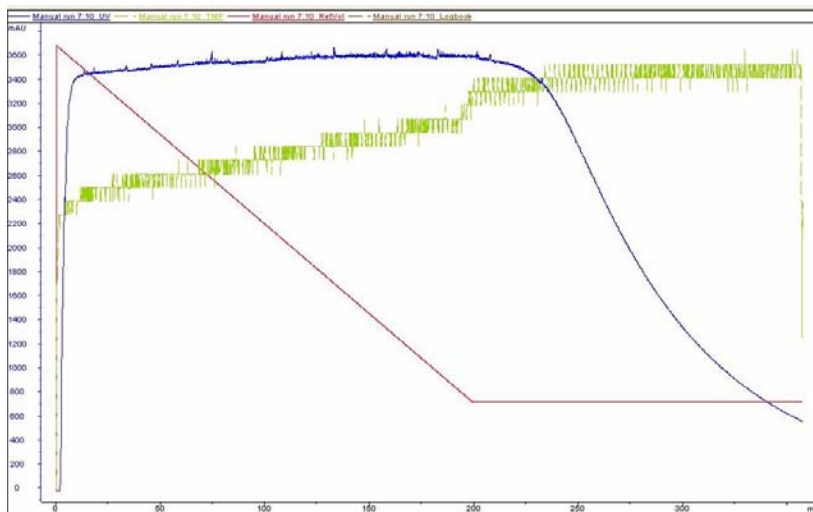


Fig 6-4. Results of a full process run to confirm recovery at maximum flux.

During concentration the retentate volume is decreasing and then it is kept constant during the diafiltration step while the UV is decreasing as the target protein is transferred to the permeate.

6.2.6 Conclusion

The clarification of the *E.coli* lysate used in this study requires membranes with relatively large pore sizes. To achieve a fast, effective and robust process for the clarification of this cell lysate containing GFP-His, a membrane pore size of 0.1 µm was used, at a shear rate of 16000 sec⁻¹ and a flux rate of 45 LMH. In some cases, when a higher recovery is desired, for example with proteins of high value, a membrane pore size of 0.2 µm may be the most appropriate choice.

Determination of the maximum feed volume for a given process time

Another critical parameter for running a successful microfiltration process is finding the right feed load to membrane area ratio for improved process economy.

The calculation of maximum feed volume is based on the selected maximum flux, the desired process time and, in the case of the example above, the 3x concentration and three wash steps.

$$\text{Max Feed Volume} = Q_{\text{max}} \times t \times m^2 \times (V_f / V_p)$$

Where:

- Q_{max} = Maximum flux rate (lmh)
- t = Target process time for scale up (h), user defined
- m^2 = Surface area of cartridge used for optimization
- V_f = Feed volume (L)
- V_p = Full process accumulated permeate volume (L)

Conclusions:

- *Perform process verification at calculated load capacity to evaluate recovery and TMP stability.*
- *Decreasing the feed volume from what was used for optimization experiments will shorten the process time with no impact on recovery.*
- *Increasing the feed volume may impact recovery and TMP stability and thus must be evaluated.*

6.3 Optimization of a concentration/diafiltration process for a BSA solution

6.3.1 Objective

The objective of this application was to optimize the conditions for a process which includes 5x concentration followed by five diafiltrations, while targeting a very high final protein concentration.

The sample was 30 mg/ml BSA (~66 kD molecular weight) in PBS buffer, and the sample volume was 250 ml. CFF experiments were carried out on ÄKTAcrossflow system using a 50 cm² Kwick Start cassette, 10 kD NMWC. The diafiltration buffer used was 50 mM acetate buffer pH 5.5.

6.3.2 Process Optimization

The strategy for process optimization was as follows:

- Membrane selection
- Optimization of the critical process parameters
- Verification of the optimized parameters in a complete run

6.3.3 Membrane selection

Membranes were screened to identify the highest membrane NMWC which retained the target molecule. The BSA recovery was calculated by measuring the absorbance at 280 nm for both the retentate and permeate. As a general rule select a membrane with a NMWC which is at least five times lower than the molecular weight of the target protein.

6.3.4 Optimization of critical process parameters

TMP excursions

A series of TMP excursions were carried out. Five TMP setpoints were tested at three crossflow rates in total recycle mode at the initial BSA concentration (30 mg/ml) and at the target BSA concentration (150 mg/ml). The TMP excursion plan is summarized in Table 6-6. The crossflow range tested was based on typical operating crossflow rates from 300 to 500 LMH, which corresponds to 25 to 42 ml/min for a 50 cm² membrane.

BSA concentration (mg/ml)	Cross flow rate (ml/min)	TMP set points (bar) in 0.5 bar increments
30 and 150	42	0.8-2.8
	33	0.8-2.8
	25	0.8-2.8

Table 6-6. TMP excursion plan for the BSA runs.

The Method Wizard in ÄKTAcrossflow software was used to create a method for running the TMP excursions. The Flux vs TMP curves were generated from the collected data using the Evaluation Wizard and the results are shown in Fig. 6-5. Runs done at 30 mg/ml BSA indicate that the TMP setpoints operate in the pressure dependent region until the TMP becomes higher than 2.3 bar, where the pressure independent region of the operation starts. As anticipated, higher crossflow rates result in higher flux because of the more efficient reduction of the gel layer at the membrane surface. At 150 mg/ml BSA, the pressure independent region of operation is more apparent. The optimal crossflow rate is that which gives the highest flux (shortest process time). However, this must be balanced against the sensitivity of the protein to shear (a higher crossflow rate results in the protein passing through the pump more times) and the disadvantage of requiring high pump capacity if scaled up. In this case, a crossflow rate of 42 ml/min was chosen.

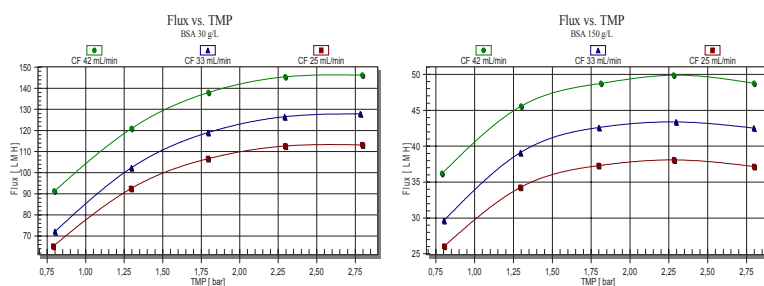


Fig 6-5. TMP excursion results. The curves were created by the ÄKTAcrossflow Evaluation Wizard from the raw data generated during the TMP scouting runs.

The optimal TMP ensures operation in the transition region between the pressure dependent and independent region for a robust process, and so a TMP of 1.8 bar was chosen.

6.3.5 Diafiltration time optimization

To minimize the diafiltration time, the optimal concentration for diafiltration was established by analysing the relationship between flux and concentration factor. A five times concentration process was run at the optimized crossflow and TMP settings, and a plot of the Flux*concentration factor versus concentration factor was generated using the Evaluation Wizard. The concentration factor that gives the highest value of the Flux*concentration factor will give the shortest diafiltration time. Fig. 6-6 shows that the diafiltration will take the same amount of time whether it is performed at a four or five times concentration. This is because the decrease in retentate volume at five times concentration is offset by the decrease in flux. However, since the buffer consumption is lower at five times concentration, as there is a smaller retentate volume to exchange, the diafiltration was performed at this concentration. In some processes it saves time to run the diafiltration step at a lower concentration and then concentrate to the desired retentate volume, but this was not the case here.

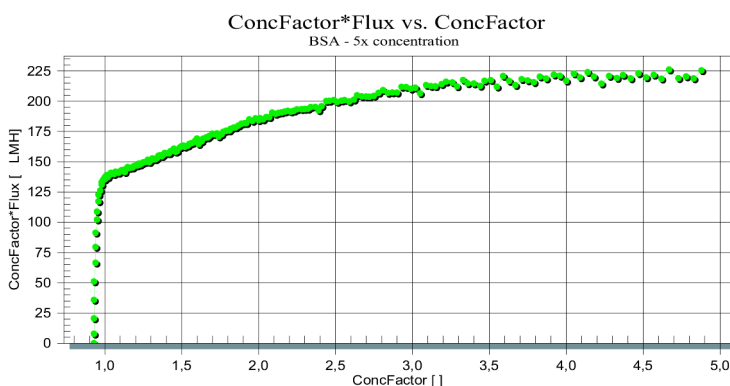


Fig 6-6. Diafiltration time optimization results.

6.3.6 Conclusion

The optimal process in this ultrafiltration application was defined by running TMP excursions and a concentration run with the optimal crossflow and TMP settings for the diafiltration time optimization. It was simple, hands free and the results were created automatically from the raw data by the software.

The optimized process can be summarized as follows:

- BSA starting concentration 30 mg/ml
- Process Crossflow rate 42 ml/min
- Process TMP 1.8 bar
- Start diafiltration at five times concentration
- BSA final concentration 150 mg/ml

The complete BSA process was run to verify the optimized parameters and some of the resulting curves are shown in Fig. 6-7.

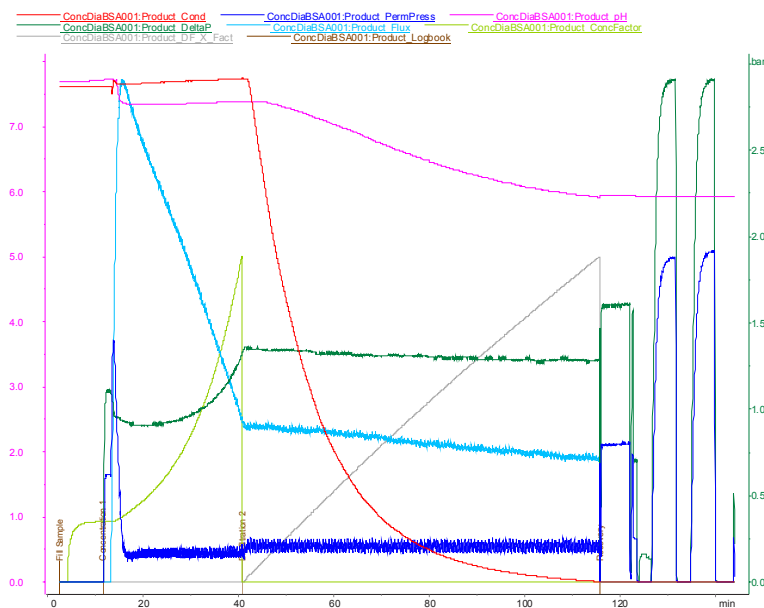


Fig 6-7. Complete BSA process with optimized parameters. During the diafiltration step the conductivity of the permeate and consequently the retentate was lowered from 16 mS/cm to 4 mS/cm and the pH was decreased from 7.4 to 5.9.

6.4 Concentration of cell culture supernatant containing IgG4

6.4.1 Objective

The objective of this application was a forty times concentration of the IgG4 solution from 0.3 mg/ml to 12 mg/ml. The sample was 2000 ml of cell culture supernatant containing 0.3 mg/ml IgG4. To keep the sample load within the recommended range (30-200 l/m²) three Kwick Start cassettes, (50 cm², 30 kD) were connected in parallel, giving a total membrane filter area of 150 cm². The sample solution was applied in fed batch mode starting with an initial volume of 50 ml solution in the reservoir and continuously feeding sample to reservoir at the same rate as the permeate was removed.

6.4.2 Process optimization

The strategy for the optimization of process parameters was similar to that of the earlier example of BSA. The IgG4 concentration in the various samples was determined using a Hi-Trap™ Protein A column.

6.4.3 Optimization of critical process parameters

Generation of TMP excursion curves

A series of TMP excursions were carried out. Five TMP setpoints were tested at three crossflow rates at the initial IgG4 concentration (0.3 mg/ml) and at the target IgG4 concentration (12 mg/ml). See Fig. 6-8. At the starting concentration the process is pressure dependent over the whole range of TMPs tested. However, at the target concentration most of the TMP values are in the pressure independent region. When analyzing the curves an optimal window of operation for TMP is found between 1.2 and 1.5 bar. The evaluation of the correlation between crossflow and flux showed that the highest flux was achieved at a crossflow rate of 125 ml/min.

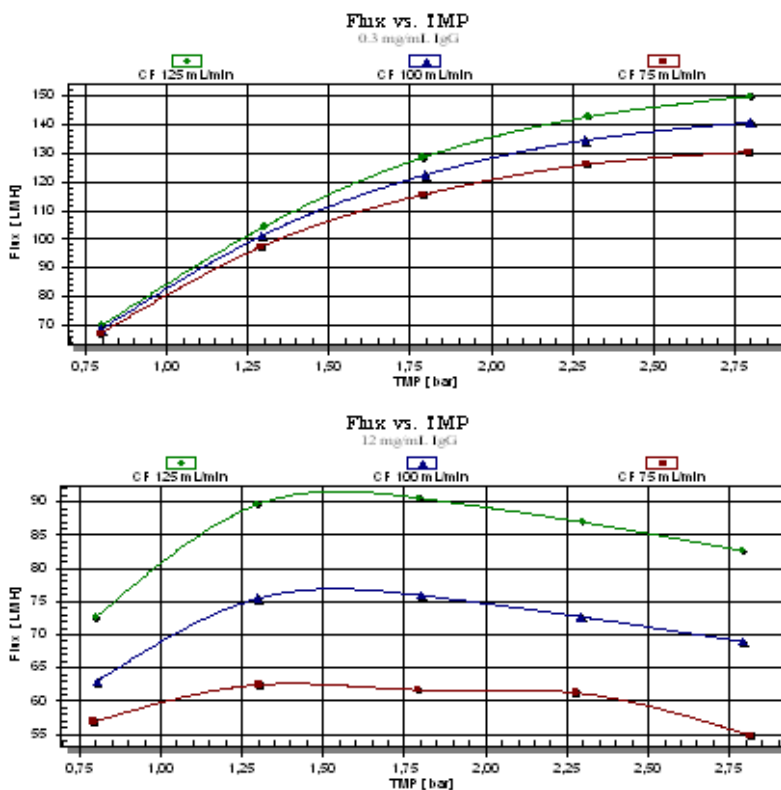


Fig 6-8. TMP excursion results for the IgG4 runs.

Determination of the optimal concentration factor for diafiltration

As with the previous ultrafiltration application using BSA, the optimal concentration for diafiltration was established by analyzing the relationship between flux and concentration factor. A forty times sample concentration was

obtained with the optimal crossflow and TMP settings and a concentration factor*Flux versus concentration factor plot was created in the Evaluation Wizard. The results are shown in Fig. 6-9. As described earlier, the highest value on the Y axis at the highest concentration factor indicates the fastest diafiltration with the lowest buffer consumption. The shortest process time and the lowest buffer consumption occurs when the diafiltration is run at the target concentration factor of 40, since the diafiltration time optimization parameter (Flux*concentration factor) is continually increasing

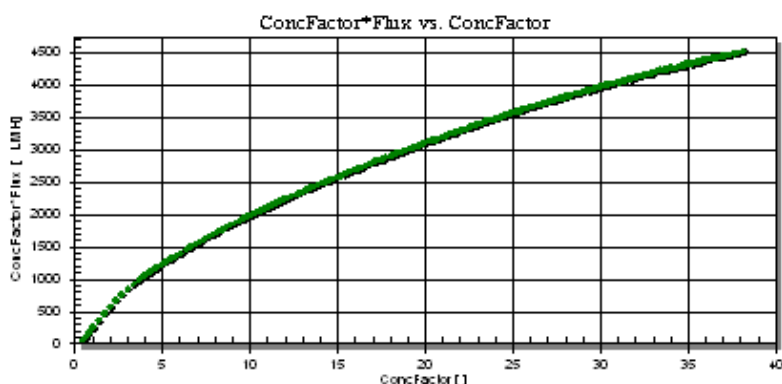


Fig 6-9. Diafiltration time optimization for the IgG4 sample.

6.4.4 Concentration and diafiltration process

The complete process for concentration and diafiltration of an IgG4 solution was run using the optimized parameters, TMP 1.3 bar and feed flow 42 ml/min. The total sample processed was 2000 ml, using three Kwick Start cassettes connected by a manifold (150 cm² total area), at constant TMP control mode. The sample was concentrated 40 times from an initial concentration of 0.3 mg/ml to 12 mg/ml and then a five times buffer exchange was performed. The collected data of various parameters are shown in Fig. 6-10. From the curves in Fig. 6-10 it can be seen how the flux decreases as the concentration factor increases during concentration. In the diafiltration step UV, the absorbance at 280 nm decreases from 2100 mAU to 40 mAU as some of the contaminants in the protein solution are washed from the retentate to the permeate.

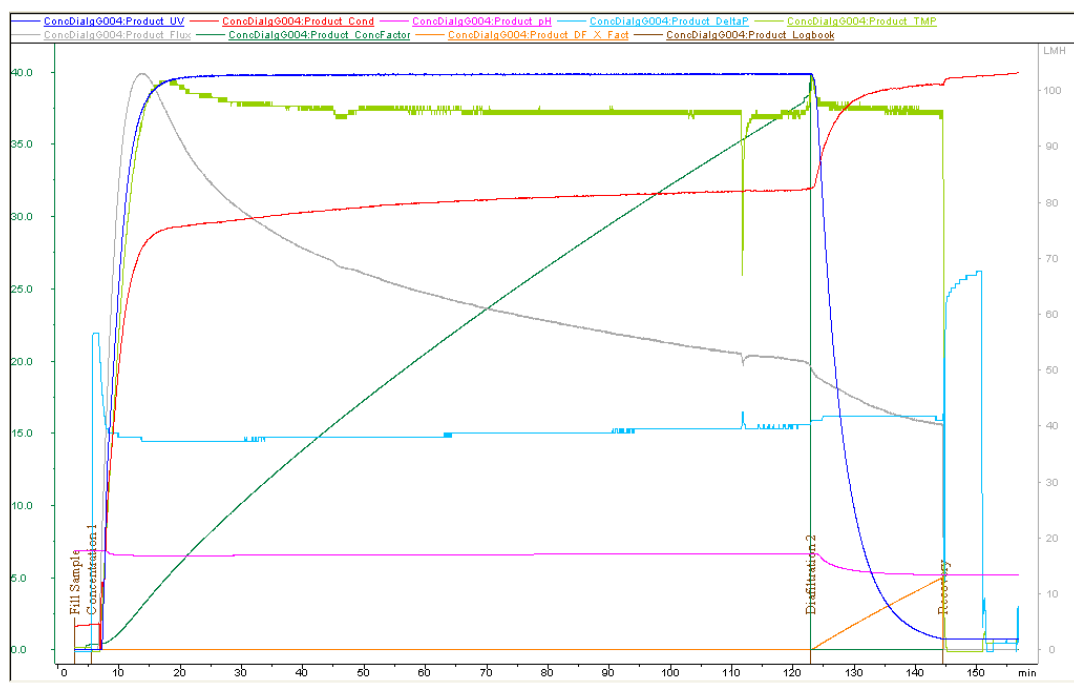


Fig 6-10. Complete concentration process of IgG4 with optimized parameters.

6.4.5 Analysis of IgG samples using a Hi-Trap Protein A column

A Hi-Trap Protein A column was used to determine the relative IgG4 concentration of the samples during the process, and the results are shown in Fig. 6-11. The chromatography runs are very reproducible for both the starting material and the concentrated IgG4 taken after diafiltration. The retention times were identical and the peak heights of the start sample and the concentrated

sample were the same after the concentrated sample had been diluted 40 times. Gel electrophoresis confirmed there was no IgG4 in the permeate.

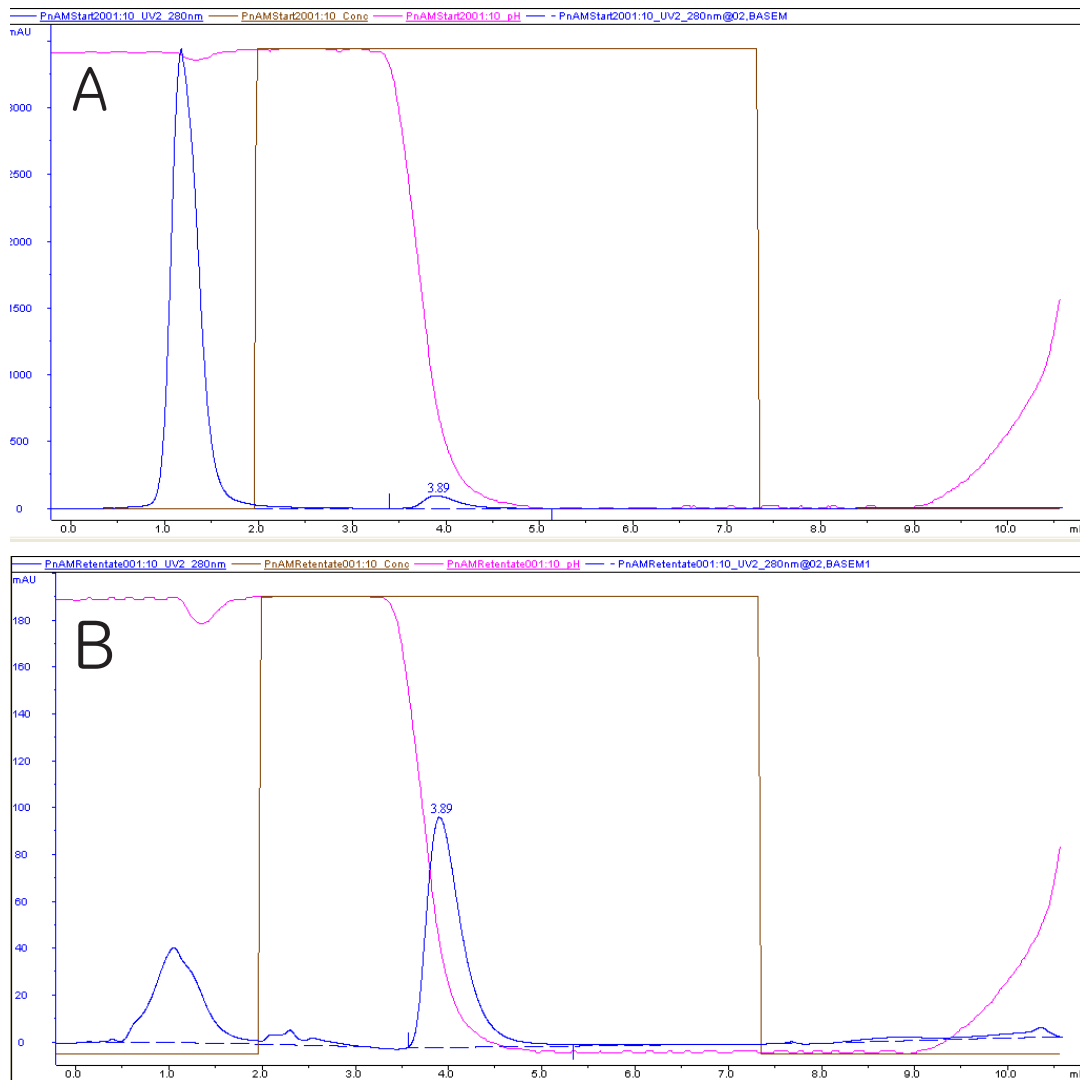


Fig 6-11. Hi-Trap column chromatography results of IgG4. The column was equilibrated with, 20 mM sodium phosphate buffer pH 7.0. 100 μ l of the samples were applied to the column at a fluid velocity of 1 ml/min. The IgG was eluted with 0.1 M sodium citrate buffer pH 3.5. (A) start material (B) concentrated material after diafiltration diluted forty times.

6.4.6 Conclusion

In this application, ÄKTAcrossflow system was used with Kwick Start cassettes to concentrate IgG4 from a clarified cell culture supernatant. The optimal process parameters were defined by running TMP excursions as well as a concentration run to find the most suitable concentration factor for performing diafiltration.

The optimized process can be summarized as follows:

- IgG4 starting concentration 0.3 mg/ml
- Process Crossflow rate 125ml/min
- Process TMP 1.3 bar
- Optimal time for diafiltration is at forty times concentration
- IgG4 final concentration 12 mg/ml

Appendix A Membrane Filters for ÄTAcrossflow system

A.1 Hollow Fiber Cartridges

Configuration	Catalogue number	Pore size	Fiber diameter (mm)	Number of fibers	Nominal surface area (cm ²)
Start AXH, 60-cm path length, ultrafiltration	UFP-3-C-H24U	3 kD	0.5	4	40
	UFP-10-C-H24U	10 kD	0.5	4	40
	UFP-30-C-H24U	30 kD	0.5	4	40
	UFP-100-C-H24U	100 kD	0.5	4	40
	UFP-300-C-H24U	300 kD	0.5	4	40
	UFP-500-C-H24U	500 kD	0.5	4	40
Start AXM, 30-cm path length, ultrafiltration	UFP-3-C-2U	3 kD	0.5	12	50
	UFP-10-C-2U	10 kD	0.5	12	50
	UFP-30-C-2U	30 kD	0.5	12	50
	UFP-100-C-2U	100 kD	0.5	12	50
	UFP-300-C-2U	300 kD	0.5	12	50
	UFP-500-C-2U	500 kD	0.5	12	50
	UFP-500-E-2U	500 kD	1.0	6	50
	UFP-750-E-2U	750 kD	1.0	6	50
Start AXM, 30-cm path length, microfiltration	CFP-1-E-2U	0.1 µm	1.0	6	50
	CFP-2-E-2U	0.2 µm	1.0	6	50
	CFP-4-E-2U	0.45 µm	1.0	6	50
	CFP-6-D-2U	0.65 µm	0.75	8	50
Cartridge kit	CFP-CELL-KIT-2U	Contains 1 each of UFP-750-E-2U, CFP-1-E-2U, CFP-2-E-2U, CFP-4-E-2U, and CFP-6-D-2U			

A.2 Membrane Cassettes for ÄKTAcrossflow system

Code number	Model number	Pore size (kD)	Membrane area (cm ²)
11-0006-02	UFEST0005050ST	5	50
11-0006-04	UFEST0010050SE	10 Select	50
11-0006-03	UFEST0010050ST	10 Select	50
11-0006-05	UFEST0030050ST	30	50
11-0006-06	UFEST0050050ST	50	50
11-0006-08	UFEST0100050ST	100	50
11-0006-61	UFESTCPAK045ST	5, 10 Select, 10, 30, 50, 100	50

Appendix B Glossary of terms

B.1 Glossary of terms

Adsorption	The binding of molecules to a surface as a result of a chemical or physical interaction between the membrane surface and the molecule.
Air diffusion rate	The rate at which air diffuses through the wetted pores of a membrane at a given differential pressure. Measuring the air diffusion rate is a method used to check the integrity of a membrane filter.
Autoclave, autoclavability	An autoclave is a device that uses saturated steam at a specified pressure over time to kill microorganisms and thus achieve sanitization or sterilization. Because many materials change properties when exposed to moisture, heat and pressure, products destined for this process must be specially engineered for autoclavability.
Back flushing, backwash	Reversing the permeate flow to mechanically clean the membrane.
Binding	The process by which some components in a feed solution adhere to the membrane. Binding can be desirable in some instances, but often, as in the case of protein, binding during sterile filtration can result in a loss of valuable product.
Biosafety tests	A class of tests that determine whether a filter's materials of construction can induce systemic toxicity, skin irritation, sensitization reaction or other biological responses. These tests are often completed by labs in vivo or in vitro. For example, United States Pharmacopoeia Class VI Plastics Test involves both the implantation and extraction of drug-product contact surfaces to demonstrate that these materials are not toxic to various mammalian cells.
Blinded	When a filter is blinded, particles have filled the pores and the flow through the filter from the feed side to the permeate side is reduced or stopped.

Bubble point	The minimum pressure required to overcome the capillary forces and surface tension of a liquid in a fully wetted membrane filter. The bubble point value is determined by observing when bubbles first begin to emerge on the permeate (downstream) side of a fully wetted membrane filter when pressurized with a gas on the feed (upstream) side of the membrane filter. By knowing the surface tension of the liquid, the pore size can be determined.
Bubble point test	The test procedure for determining the bubble point of the largest pores in a microfiltration membrane.
Buffer exchange	Filtration process used for the removal of smaller ionic solutes, whereby the feed solution is washed, usually repeatedly, and one buffer is removed and replaced with an alternative buffer.
Cartridge or cartridge filter	A filtration or separation device having a membrane encapsulated within a housing. In the case of hollow fiber cartridges, the housing normally has feed and permeate ports and, in the case of cross flow filters, a retentate port. All of these ports may be used to control the flow parameters of fluid into and out of the housing and through the membrane.
Cassette	A device used for cross flow filtration, typically in a rectangular form comprised of stacked flat sheets of membrane integrally bonded together. Most cassettes are typically designed to fit into a standard cassette holder where the feed, permeate and retentate ports mate with appropriate fittings on the cassette holders.
Cell harvesting	The process of concentrating (dewatering) the cell mass after fermentation. Cell slurries in excess of 70% wet cell weight are achievable. The cells may also be washed to prepare them for further processing, such as freezing or lysing. Unlike clarification processing, with cell harvesting, the cells are the target material.
Channel height	The height of the path that the feed/retentate solution must pass through for a flat membrane cassette.
Channel length	See flow path length.

Chemical compatibility	The ability of the components of a filter to resist chemicals that can influence the filter's performance. For example, some chemicals could cause the filter to swell or dissolve filter components. Repeatable performance requires that filters are resistant to all the chemicals that they are exposed to at a given concentration, temperature and total exposure time.
Clean-in-place (CIP)	The process of cleaning a filtration device without removing it from its filtration system. CIP processes can remove proteins, lipids, cell debris, micro-organisms, and other contaminants.
Composite membrane	A membrane that is made up of two or more layers that are usually chemically or structurally different.
Concentrate	Also called retentate. The part of the process solution that does not pass through a cross flow membrane filter.
Concentration	Cross flow filtration process in which the components that do not pass through the membrane remain in the feed loop and therefore increase in concentration as permeate leaves the system.
Concentration factor	The concentration factor equals the ratio of the initial feed volume to retentate volume after separation. For example, if the initial feed volume is 100 l and the final retentate volume is 20 l, the concentration factor is 5x.
Concentration polarization	The buildup of molecules of dissolved substances (solutes) on the surface of the membrane filter during filtration. The concentration polarization layer increases resistance to permeate flow and reduces the permeate flux, thus decreasing filtration efficiency.
Cross flow filtration (CFF)	Also called tangential flow filtration. In cross flow filtration, the feed solution flows parallel to the surface of the membrane. Driven by pressure, some of the feed solution passes through the membrane filter. Most of the solution is circulated back to the feed tank. The movement of the feed solution across the membrane surface helps to remove the buildup of materials on the surface.

Cross flow rate	Also called retentate flow rate. The flow rate of feed solution that flows across the surface of the filter and exits the filter as retentate. Higher cross flow rates help "sweep away" the debris that forms on the surface of the filter. Cross flow rate is most often measured at the retentate outlet.
Cutoff	See Molecular weight cutoff (MWCO) and Nominal molecular weight cutoff (NMWC).
Dead-ended filtration	See normal flow filtration.
ΔP	ΔP , or pressure differential between the feed and retentate lines. The ΔP equals the feed pressure minus the retentate pressure.
Depth filter	A thick filter that captures contaminants within its pore structure using entrapment and adsorption. A membrane filter primarily captures contaminants on its surface.
Depyrogenate	The removal or decomposition of pyrogens (lipopolysaccharides, endotoxins) from a process solution.
Diafiltration	A unit operation that incorporates ultrafiltration membranes to remove salts or other microsolute from a solution. Small molecules are separated from a solution while retaining larger molecules in the retentate. Microsolute are generally so easily washed through the membrane that for a fully permeated species about three volumes of diafiltration solution will eliminate 95%-99% of the microsolite.
Diafiltration exchange factor	Diafiltration exchange factor = Diafiltration buffer volume / Sample volume
Dialysis	Removal of small molecules from a solution of macromolecules by allowing them to diffuse through a semi-permeable membrane into water or a buffer solution. This osmotic pressure separations method is controlled by the concentration gradient of salts across the membrane.
Differential pressure	See ΔP .

Diffusion	Movement of liquid or gas particles from a region of higher concentration to a region of lower concentration.
Direct flow filtration	See normal flow filtration.
Downstream processing	Starting with a feed stream free of cells and cell debris, the purification sequences involving chromatography and membrane separations to achieve final product purity.
Effective filtration area	In a membrane separations device, the active area of the membrane exposed to flow.
Endotoxin	The outer cell wall of gram-negative bacteria, also known as LPS (lipopolysaccharides) and pyrogens.
Ethylene oxide (EtO) sterilization	A sterilization process still common for biomedical products, in which product is subjected to steam and highly toxic ethylene oxide gas. Because many materials change properties when exposed to moisture and EtO by-products, products destined for this process must be specially engineered for EtO sterilization.
Extractables	Substances that may dissolve or leach from a membrane device during filtration and contaminate the process solution. For example, these might include wetting agents in the membrane, membrane cleaning solutions or substances from the materials used to encase the membrane.
Feed	Material or solution that is introduced into a membrane separation system.
Feed pressure	The pressure measured at the inlet port of a cartridge or cassette.
Filter area	The surface area of filter media inside a separation device.
Filter efficiency	Filter efficiency represents the percentage of a given size particle removed from the fluid by the filter.
Filtrate	Also called permeate. The portion of the process fluid that passes through the membrane.

Flow path length, nominal flow path length	The total length that a feed solution travels from inlet to outlet. Flow path length is an important parameter to consider when doing any process development, system design or scale-up or scale-down experiments. The flow path length and other fluid channel geometries such as lumen diameter or channel height can affect the fluid dynamics of the system and will directly affect pump requirements and differential pressure of the filtration step.
Flux	Flux represents the volume of solution flowing through a given membrane area during a given time. Expressed as LMH (liters per square meter per hour).
Fouling	A build up of material on the membrane surface that reduces the filtration rate. Unlike the gel layer, this material is not redispersed in the bulk stream by higher shear rates.
Gel layer	During the filtration process, the thin layer of particles or molecules that may build up at the membrane surface is called the gel layer. It is also referred to as the concentration polarization layer. High TMP can be the result of an increase in the thickness of the gel layer. Gel layer formation can negatively impact the filtration process by reducing flux and inhibiting passage through the membrane. Operating at a higher shear rate may reduce the thickness of the gel layer.
Hold-up volume	Quantity of fluid remaining within the system after the filtration step is complete.
Hollow fiber	A tube made of membrane. When sealed inside a cross flow cartridge, the feed stream flows into the inner diameter of one end of the hollow fiber and the retentate (the material that does not permeate through the walls of the hollow fiber) flows out the other end. The material that passes through the membrane (walls of the hollow fiber) is called the permeate.
Housing	The mechanical structure that surrounds and supports the membrane or filter element. The housing normally has feed, retentate and permeate ports that direct the flow of process fluids into and out of the filter assembly.

Hydrophilic	Filters that wet easily with water and work well with aqueous solutions.
Hydrophobic	Filters that do not wet easily with water but typically do wet easily with nonpolar solvents such as alcohol. Once wetted a hydrophobic filter may be used to process many aqueous solutions.
<i>In vitro</i>	An experiment performed in a test tube, Petri dish or other lab apparatus with parts of a living organism, such as testing a drug with tissue samples. From Latin, meaning "in glass".
<i>In vivo</i>	An experiment performed using a living organism. From Latin, meaning "in live [subjects]".
Inlet pressure	The pressure of a fluid at the feed port of a separation device.
Isoelectric point	The pH at which a protein carries no net electric charge.
Lumen	The inner, open space of a hollow fiber.
Macrovoid	A generally undesirable open space in the substructure of a membrane filter that is appreciably larger than the average pore size. Macrovoids can lead to pinhole defects resulting in unwanted passage that directly affects final product yield. Macrovoids can also affect the overall membrane strength and thus the device's ability to maintain integrity under pressure.
Media exchange	A filtration step used during aseptic cell culturing to remove growth media or fermentation broth so that fresh growth media can be added to the bioreactor.
Membrane	A thin layer of a highly engineered material with controlled pore size and used to separate particles, biological matter and molecules from a solution.
Membrane recovery	The degree to which the original performance of a membrane can be restored by cleaning.
Microfiltration	The process of removing particles from a liquid by passing it through a porous membrane under pressure. Microfiltration usually refers to removing submicron-size particles.
Micron (micrometer, μm)	One one-millionth of one meter.

Microporous membrane	A thin, porous film or hollow fiber having pores ranging from 0.1 to 10 µm. Cross flow microfilters typically have pores ranging from 0.1 to 1.0 µm.
Minimum process volume	Also called minimum operating volume. The least amount of fluid able to be handled effectively by a filtration system.
Molecular weight	Mass of one molecule of a nonionic substance in atomic mass units.
Molecular weight cut off (MWCO)	See NMWC.
Nanofiltration	Separation processes targeted for solutes having molecular weights from 500 to 1,000D. See Molecular weight cut off (MWCO).
Nominal filter rating	A rating that indicates the percentage of particles of a specific size or molecules of a specific molecular weight that will be removed by a filter. No industry standard exists; hence the ratings from manufacturer to manufacturer are not always comparable.
Nominal molecular weight cut off (NMWC)	The size designation in Daltons (D) for ultrafiltration membranes. No industry standard exists; hence the NMWC ratings of different manufacturers are not always comparable.
Normal flow filtration	Also called dead-ended filtration. In normal flow filtration, liquid flows perpendicular to the filter media, and all of the feed passes through.
Normalized water permeability (NWP)	The water flux at 20 °C divided by pressure. Common units LMH/psi, LMH/bar.
Particle size distribution	The distribution of particle sizes (number or weight fraction) in a fluid.
Permeate	Also called filtrate. Any components of the feed solution that passes through the membrane.
pH	Negative logarithm of the hydronium ion (H_3O^+) concentration in an aqueous solution. Indicates the acidity or alkalinity of a substance.

Programmable logic controller (PLC)	A device for industrial control. Types of operations common to PLCs are polling or checking sensors and activating/deactivating valves and switches compared against programmed presets or default levels.
Pleating	Folding flat sheet filter media to increase the surface area that can be fitted into a given separation device. Generally used in dead-end filtration.
Pore size distribution	The range of pore sizes in a membrane. The tighter the pore size distribution, the better control one has over the filtration process.
Porosity	A measurement of the open space in a membrane. The higher the membrane porosity, the more pores there are, and hence a higher flow rate is anticipated.
Pressure drop	The difference in pressure between two points.
Protein passage	The passing of protein from the feed stream into the permeate stream.
Pyrogen	A substance that produces a fever within a warm-blooded animal when injected into the bloodstream. Filtration materials of construction that come in contact with injectable liquids must meet pyrogenicity standards.
Recovery	Percentage of the target substance that can be collected in the retentate or permeate solution after processing.
Retentate	The portion of the feed solution that does not pass through a cross flow membrane filter. Any component that does not pass through the membrane flows out of the filter and back to the feed container. Any components in the return line is called retentate.
Retention	The ability of a separation device to retain an entity of a given size.
Reverse osmosis	Type of crossflow filtration used for removal of very small solutes (<1,000 Daltons) and salts. It uses a semi-permeable membrane under high pressure to separate water from ionic materials. High pressure is necessary to overcome the natural osmotic pressure created by the concentration gradient across the membrane.

Sanitization	A cleaning process that destroys most living microorganisms, reducing the microbial population to an acceptable, predetermined level.
Separation	Dividing a liquid or gas feed stream into separate components.
Shear rate	A ratio of velocity and distance expressed in units of sec^{-1} . The shear rate for a hollow fiber cartridge is based on the flow rate through the fiber lumen.
Sieving	Removal of particles from a feed stream as a result of entrapment within the depth of the membrane pore structure.
Steam-in-place (SIP)	The process of sterilizing a device, such as a hollow fiber cartridge, with steam, without removing the device from the separation system.
Size exclusion	Mechanism for removing particles from a feed stream based strictly on the size of the particles. Retained particles are held back because they are larger than the pore opening.
Solute	An ionic or organic compound dissolved in a solvent.
Starling flow	A portion of permeate that is driven back through the membrane in the reverse direction near the outlet of the cartridge, due to the high permeability of these membranes in the presence of permeate pressure. This phenomenon is most often associated with the operation of microfiltration membranes using permeate flow control.
Sterilization	A process that removes/destroys all microorganisms from a solution or a filtration system. See Autoclave, EtO Ethylene oxide sterilization, Gamma sterilization.
Surface filter	A filter in which particles larger than the pores are retained on the surface of the filter.
Tangential flow filtration	See cross flow filtration.
Thermal stability	The ability of a membrane and filtering device to maintain its performance during and after exposure to excursions of temperature, such as the elevated temperatures experienced during autoclaving or steam sterilization.

Throughput	The volume of solution that will pass through a separation device before the permeate output drops to an unacceptable level.
Transmembrane pressure (TMP)	The pressure differential between the upstream (feed) and downstream (permeate) sides of a membrane. It is calculated by: $TMP = (P_{\text{Feed}} + P_{\text{Retentate}})/2 - P_{\text{Permeate}}$
Turbidity	Measure of relative clarity of a liquid. Measurements are based on the amount of light transmitted through a sample. The more light that is scattered by fine solids or colloids, the less clear (and more turbid) the solution. Often reported in NTU (nephelometric turbidity unit).
Ultrafiltration	The separation of macromolecules based on their molecular weight or size.
Upstream	The feed side of a separation process.
Upstream processing	Cellular separations including cell lysates, cell harvesting, clarification and cell culture perfusion.
Viral clearance	The removal of viral contamination using methods such as, filtration, chromatography, heat and low pH.
Viscosity	A measurement of a fluid's resistance to shear. A slow-flowing liquid such as gear oil has a higher viscosity than a free-flowing liquid such as water. In a given separation process, higher-viscosity, Newtonian fluids are operated at a lower flow rate through a cartridge than do lower-viscosity fluids.
Void volume	Quantity of fluid required to completely fill a section of piping. Also, the amount of open space within membrane filter media.
Water flux	Measurement of the amount of water that flows through a given membrane surface area in a set time. See also flux. The water flux test is commonly used to assess cleaning efficacy.
Yield	The amount of particulates or molecules of interest (product) that can be recovered from the cross flow filtration process. Also called recovery.

Appendix C Shear effects on proteins and cells

In a crossflow filtration system the protein solution or cell suspension is recirculated over the filter membrane, and thus passes through the pump many times. It is therefore essential that the pump causes as little protein aggregation or cell disruption as possible.

In order to measure the effects of shear induced by different pump designs, a series of studies was made comparing ÄKTAcrossflow P984 pumps and traditional pumps and the influence of a piston rinsing system, on protein aggregation and cell disruption.

C.1 Shear studies on protein solutions

A P984 pump fitted with ÄKTA™ sanitary tubing (i.d. 3mm, max flow rate 800ml/min) was compared to a peristaltic pump fitted with Marprene II #25 hose (i.d. 4.8mm, max flow rate 690 ml/min). In these studies a rotary piston pump, with no piston sealing or rinsing system, was also tested.

C.1.1 Piston rinsing system

Fig. C-1 shows the results of a pump rinsing system on protein aggregation as measured by UV absorbance at 620nm, using ÄKTAcrossflow pump P984. The rinsing system reduces the friction between the piston and the cylinder wall and reduces protein aggregation due to shear. The choice of rinsing fluid is important (Fig. C-2). Sodium hydroxide dissolves the aggregates, and ethanol reduces the viscosity making the rinsing more effective. The recommended rinsing solution for ÄKTAcrossflow system is 10 mM sodium hydroxide in 20% ethanol.

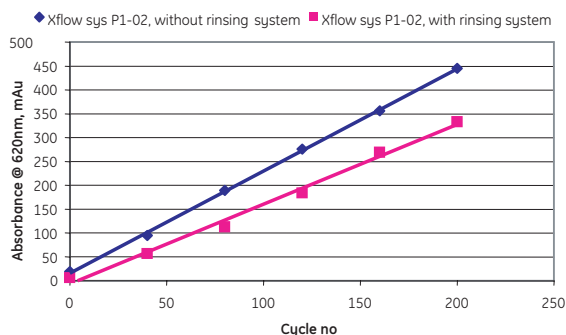


Fig C-1. Influence of using a piston rinsing system on protein aggregation using P984 pump

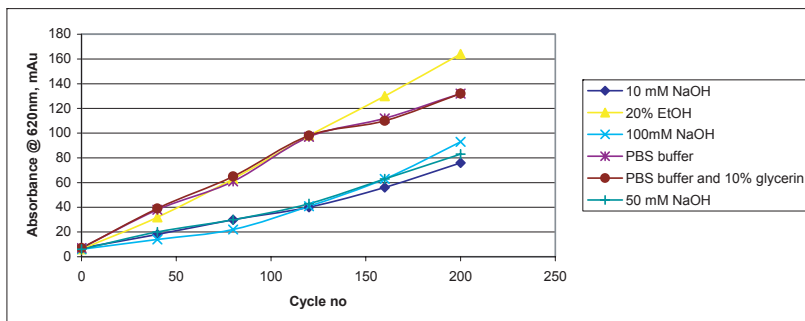


Fig C-2. Influence of rinsing fluids on protein aggregation

C.1.2 Comparison of different pump types

Human IgG, 20 mg/ml in 100 mM PBS buffer pH 7.0 containing 20 mg/ml glycine was used as the sample, and the absorbance at 620 nm measured as a function of the number of cycles (Fig. C-3). Protein aggregation was highest when using the rotary piston pump. P984 pump showed the lowest protein aggregation of all pumps.

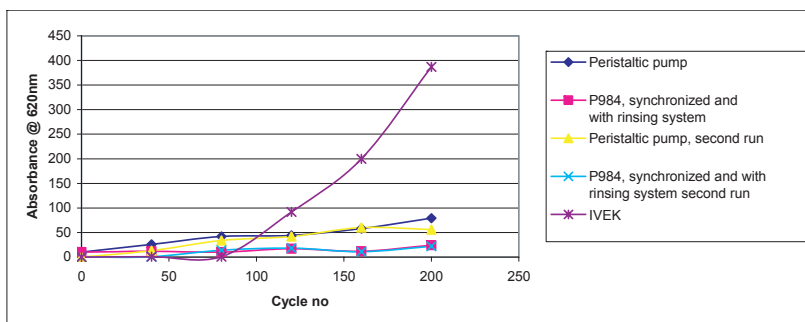


Fig C-3. Comparison of different pump designs and the formation of protein aggregates as a function of cycle numbers.

C.2 Shear studies on cell suspensions

A P984 pump fitted with ÄKTA sanitary tubing (i.d. 3mm, max flow rate 800ml/min) was compared to a peristaltic pump fitted with Marprene II #25 hose (i.d. 4.8mm, max flow rate 690 ml/min), using a sample of wild type CHO cells. Cell integrity was measured as a function of LDH activity and cell viability by counting free nuclei. In addition, temperature and cell densities were measured. A control sample was kept in an end over end mixer at low speed to monitor the natural increase in LDH activity. A sample of the cell suspension was homogenized three times at 800 bar to establish the LDH maximum value.

C.2.1 Comparison of pump types

Compared with the peristaltic pump, the P984 pump showed a higher cell viability, a higher cell count, a lower increase in temperature and a lower increase in LDH activity (Fig. C-4).

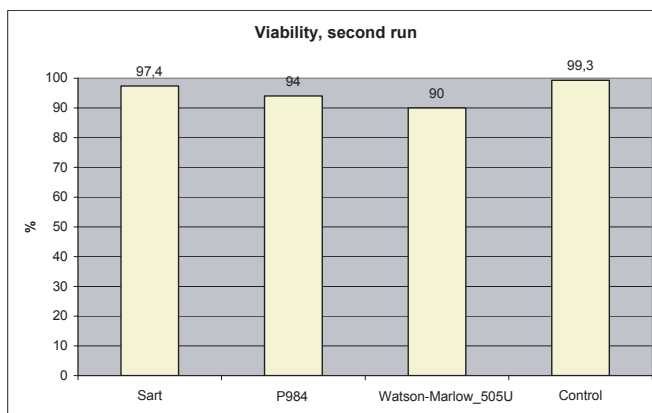


Fig C-4. 400 ml of CHO cell suspension was recirculated for 370 cycles at 400 ml/min for each pump. Measurements were made of cell viability, cell density, LDH and temperature.

The results of a comparison of a stand alone P984 pump and ÄKTAcrossflow system using the criteria of cell viability and cell density is summarized in Table C-1. The results show that cells are treated very gently in ÄKTAcrossflow system resulting in high cell density and viability and low levels of LDH.

Cell count in Nigrosin BB1744		
Sample	Viability (%)	Cell density (10 ⁶ /ml)
Starting CHO cells	98.5	1.3
Control	99.7	0.99
CHO cells after 400 cycles in P984	93.4	0.73
CHO cells after 400 cycles in ÄKTAcrossflow system	98.8	0.83
CHO cells after 200 minutes in a tank fitted with magnetic stirrer	97.2	0.93

Table C-1. 140 ml of CHO cell suspension was recirculated for 400 cycles at 280 ml/min. In addition a CHO cell suspension sample was kept in a recirculation tank with a magnetic stirrer to monitor the effect of stirring on cell integrity.

C.3 Conclusion

A comparison of shear forces induced by ÄKTAcrossflow P984 and other pumps clearly shows that ÄKTAcrossflow P984 is a much gentler pump. It is the pump of choice for CFF applications with protein solutions and cell suspensions, causing less protein aggregation and cell disruption.

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